TITLE OF INVENTION

TRANSFERRIN RECEPTOR GENES

FIELD OF INVENTION

The present invention is related to the molecular cloning of genes encoding transferrin receptor and in particular to the cloning of transferrin receptor genes from *Haemophilus influenzae*.

REFERENCE TO RELATED APPLICATION

~~This application is a continuation-in-part of~~ copending United States Patent Application No. 08/483,577, filed June 7, 1995, which itself is a continuation-in-part of copending United States Patent Application No. 08/337,483 filed November 8, 1994, which itself is a continuation-in-part of copending United States Patent Application No. 08/175,116, filed December 29, 1993 (now abandoned), which itself is a continuation-in-part of copending United States Patent Application No. ~~08/148,968 filed November 8, 1993 (now abandoned).~~

BACKGROUND OF THE INVENTION

Encapsulated *Haemophilus influenzae* type *b* strains are the major cause of bacterial meningitis and other invasive infections in young children. However, the non-encapsulated or non-typable *H. influenzae* (NTHi) are responsible for a wide range of human diseases including otitis media, epiglottitis, pneumonia, and tracheobronchitis. Vaccines based upon *H. influenzae* type b capsular polysaccharide conjugated to diphtheria toxoid (Berkowitz *et al.*, 1987. Throughout this application, various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosures of these references are hereby incorporated

by reference into the present disclosure), tetanus toxoid (Classon *et al.*, 1989 and US patent 4,496,538), or *Neisseria meningitidis* outer membrane protein (Black *et al.*, 1991) have been effective in reducing *H. influenzae* type b-induced meningitis, but not NTHi-induced disease (Bluestone, 1982).

Otitis media is the most common illness of early childhood with 60-70% of all children of less than 2 years of age experiencing between one and three ear infections. Chronic otitis media is responsible for hearing, speech and cognitive impairments in children. *H. influenzae* infections account for about 30% of the cases of acute otitis media and about 60% of chronic otitis media. In the United States alone, treatment of otitis media costs between 1 and 2 billion dollars per year for antibiotics and surgical procedures such as tonsillectomies, adenoidectomies and insertion of tympanostomy tubes. Furthermore, many of the causative organisms of otitis media are becoming resistant to antibiotic treatment. An effective prophylactic vaccine against otitis media is thus desirable. Non-typable strains of *H. influenzae* are also important pathogens responsible for pneumonia in the elderly and other individuals who are particularly susceptible to respiratory infections. There is thus a need for antigens from *H. influenzae* which are useful as components in immunogenic preparations that provide protection against the many serotypes of *H. influenzae*.

Iron is an essential nutrient for the growth of many bacteria. Several human pathogens, such as *H. influenzae*, *Branhamella catarrhalis*, *N. meningitidis*, *N. gonorrhoeae* and non-pathogenic commensal *Neisseria* strains, can utilize human transferrin as an iron source (Schryvers, 1988; Schryvers and Lee, 1989; Mickelsen and Sparling, 1981). The bacterial transferrin receptor (TfR) is composed of two chains, Tbp1 and Tbp2. In

strains of *H. influenzae*, the molecular weight of Tbp1 is approximately 100,000, whereas the molecular weight of Tbp2 is variable, ranging from 60,000 to 90,000, depending upon the strain (Schryvers and Gray-Owen, 1992; Holland *et al.*, 1992). Expression of *H. influenzae* transferrin receptor is thought to be iron-and/or hemin-regulated (Morton et al., 1993) and a putative *fur*-binding site (Braun and Hantke, 1991) has been identified upstream of *tbp2*. This sequence is found in the promoter region of genes which are negatively regulated by iron, including *N. meningitidis* TfR (Legrain et al., 1993). The promoter is followed by the *tbp2* and *tbp1* genes, an arrangement found in other bacterial TfR operons (Legrain et al, 1993; Wilton et al., 1993). Antibodies which block the access of the transferrin receptor to its iron source may prevent bacterial growth. In addition, antibodies against TfR that are opsonizing or bactericidal may also provide protection by alternative mechanisms. Thus, the transferrin receptor, fragments thereof, its constituent chains, or peptides derived therefrom are vaccine candidates to protect against *H. influenzae* disease. Mice immunized with *N. meningitidis* TfR proteins in Freund's adjuvant were protected from homologous challenge and the anti-TfR antisera were bactericidal and protective in a passive transfer assay (Danve et al., 1993). Pigs immunized with recombinant *A. pleuropneumoniae* Tbp2 were protected against homologous challenge but not heterologous challenge (Rossi-Campos et al., 1992). These data indicate the efficacy of TfR-based vaccines in protection from disease. It would be desirable to provide the sequence of the DNA molecule that encodes transferrin receptor and peptides corresponding to portions of the transferrin receptor and vectors containing such sequences for diagnosis, immunization and the generation of diagnostic and immunological reagents.

Poliovirus is an enterovirus, a genus of the family Picornaviridae. There are three distinct serotypes of the virus, and multiple strains within each serotype. Virulent strains are causative agents of paralytic poliomyelitis. Attenuated strains, which have reduced potential to cause paralytic disease, and inactivated virulent strains, are used as vaccines. Infection with the virus induces long-lasting, protective, mucosal immunity. Inoculation with inactivated poliovirus vaccines can also induce a mucosal immune response.

The structure of poliovirus is known, and is highly conserved among strains and serotypes. The structures of several other picornaviruses (viruses belonging to genera of the family Picornaviridae) have also been determined, and have been shown to be closely related to the structure of poliovirus. It is possible to express foreign epitopes on the capsid of polioviruses (Murdin et al, 1992) and this work has been extended to other picornaviruses. Epitopes which have been expressed are usually short, well defined, contiguous epitopes, and most have been expressed within poliovirus neutralisation antigenic site I (NAgI) or the equivalent site on other picornaviruses. This site includes the loop linking beta strands B and C (the BC loop) of poliovirus capsid protein VP1. The BC loop of VP1 is a surface-exposed loop of nine amino acids which can be replaced and extended with at least twenty-five heterologous amino acids (Murdin et al, 1991). Hybrid or chimeric polioviruses expressing transferrin receptor epitopes, which grow to a high titre and are immunogenic, would be useful as vaccines and as tools for the generation of immunological reagents.

SUMMARY OF THE INVENTION

The present invention is directed towards the provision of purified and isolated nucleic acid molecules

encoding a transferrin receptor of a strain of *Haemophilus* or a fragment or an analog of the transferrin receptor protein. The nucleic acid molecules provided herein are useful for the specific detection of strains of *Haemophilus*, and for diagnosis of infection by *Haemophilus*. The purified and isolated nucleic acid molecules provided herein, such as DNA, are also useful for expressing the TfR genes by recombinant DNA means for providing, in an economical manner, purified and isolated transferrin receptor subunits, fragments or analogs thereof. The transferrin receptor, subunits or fragments thereof or analogs thereof, as well as nucleic acid molecules encoding the same and vectors containing such nucleic acid molecules, are useful in immunogenic compositions against diseases caused by *Haemophilus*, the diagnosis of infection by *Haemophilus* and as tools for the generation of immunological reagents. Monoclonal antibodies or mono-specific antisera (antibodies) raised against the transferrin receptor protein produced in accordance with aspects of the present invention are useful for the diagnosis of infection by *Haemophilus*, the specific detection of *Haemophilus* (in for example *in vitro* and *in vivo* assays) and for the treatment of diseases caused by *Haemophilus*.

Peptides corresponding to portions of the transferrin receptor or analogs thereof are useful immunogenic compositions against disease caused by *Haemophilus*, the diagnosis of infection by *Haemophilus* and as tools for the generation of immunological reagents. Monoclonal antibodies or antisera raised against these peptides, produced in accordance with aspects of the present invention, are useful for the diagnosis of infection by *Haemophilus*, the specific detection of *Haemophilus* (in, for example, *in vitro* and *in vivo* assays) and for use in passive immunization as a treatment of disease caused by *Haemophilus*.

In accordance with one aspect of the present invention, there is provided a purified and isolated nucleic acid molecule encoding a transferrin receptor protein of a strain of *Haemophilus*, more particularly, a strain of *H. influenzae*, specifically a strain of *H. influenzae* type b, such as *H. influenzae* type b strain DL63, Eagan or MinnA, or a non-typable strain of *H. influenzae*, such as *H. influenzae* strain PAK 12085, SB33, SB12, SB29, SB30 or SB32, or a fragment or an analog of the transferrin receptor protein.

In one preferred embodiment of the invention, the nucleic acid molecule may encode only the Tbp1 protein of the *Haemophilus* strain or only the Tbp2 protein of the *Haemophilus* strain. In another preferred embodiment of the invention, the nucleic acid may encode a fragment of the transferrin receptor protein of a strain of *Haemophilus* having a conserved amino acid sequence which is conserved among bacteria that produce transferrin receptor protein. Such conserved amino acid sequence may have an amino acid sequence contained within the amino acid sequence of the peptides shown in Tables 2 and 3 below for *Haemophilus influenzae* type b strain Eagan as well as corresponding peptides of other strains of *Haemophilus influenzae*.

In another aspect of the present invention, there is provided a purified and isolated nucleic acid molecule having a DNA sequence selected from the group consisting of (a) any one of the DNA sequences set out in Figure 3, 4, 5, 6, 7, 8, 9, 10 or 11 (SEQ ID NOS: 1, 2, 3, 4, 105, 108, 110, 112, 114) or the complementary DNA sequence of any one of said sequences; (b) a DNA sequence encoding one of the amino acid sequences set out in Figure 3, 4, 5, 6, 7, 8, 9, 10, 11 or 31 (SEQ ID NOS: 5, 6, 7, 8, 9, 10, 11, 12, 106, 107, 109, 111, 113, 115) or the complementary DNA sequence thereto; and (c) a DNA sequence which hybridizes under stringent conditions to

any one of the DNA sequences defined in (a) or (b). The DNA sequence defined in (c) preferably has at least about 90% sequence identity with any one of the DNA sequences defined in (a) and (b).

In an additional aspect, the present invention includes a vector adapted for transformation of a host, comprising a nucleic acid molecule as provided herein. The vector may be one having the characteristics of plasmid DS-712-1-3 having ATCC accession number 75603 or plasmid JB-1042-7-6 having ATCC accession number 75607.

The plasmids may be adapted for expression of the encoded transferrin receptor, fragments or analogs thereof, in a heterologous or homologous host, in either a lipidated or non-lipidated form. Accordingly, a further aspect of the present invention provides an expression vector adapted for transformation of a host comprising a nucleic acid molecule as provided herein and expression means operatively coupled to the nucleic acid molecule for expression by the host of the transferrin receptor protein or the fragment or analog of the transferrin receptor protein. In specific embodiments of this aspect of the invention, the nucleic acid molecule may encode substantially all the transferrin receptor protein, only the Tbp1 protein or only the Tbp2 protein of the *Haemophilus* strain. The expression means may include a nucleic acid portion encoding a leader sequence for secretion from the host of the transferrin receptor protein or the fragment or the analog of the transferrin receptor protein. The expression means also may include a nucleic acid portion encoding a lipidation signal for expression from the host of a lipidated form of the transferrin receptor protein or the fragment or the analog of the transferrin receptor protein. The expression plasmid may have the identifying characteristics of plasmid JB-1468-29, JB-1600-1 or JB-1424-2-8. The host may be selected from, for example,

Escherichia coli, *Bacillus*, *Haemophilus*, fungi, yeast or baculovirus and Semliki Forest virus expression systems may be used.

In an additional aspect of the invention, there is provided a transformed host containing an expression vector as provided herein. Such host may selected from JB-1476-2-1, JB-1437-4-1 and JB-1607-1-1. The invention further includes a recombinant transferrin receptor protein or fragment or analog thereof producible by the transformed host.

As described in more detail below, there has been produced Tbp1 and Tbp2 protein receptors separate from each other. Further aspects of the present invention, therefore, provide an isolated and purified Tbp1 protein of a strain of *Haemophilus* free from the Tbp2 protein of the *Haemophilus* strain and an isolated and purified Tbp2 protein of a strain of *Haemophilus* free from the Tbp1 protein of the *Haemophilus* strain. The *Haemophilus* strain may be *H. influenzae* type b or a non-typable strain of *H. influenzae*.

The present invention further provides synthetic peptides corresponding to portions of the transferrin receptor. Accordingly, in a further aspect of the invention, there is provided a synthetic peptide having no less than six amino acids and no more than 150 amino acids and containing an amino acid sequence corresponding to a portion only of a transferrin receptor protein of a strain of bacteria or of an analog the transferrin receptor protein. The bacterial strain preferably is a *Haemophilus* strain, particularly a *H. influenzae* strain, specifically a strain of *H. influenzae* type b or a non-typable strain of *H. influenzae*.

The peptides provided herein may comprise an amino acid sequence which is conserved among bacteria that produces transferrin receptor protein, including strains of *Haemophilus*. The peptide may include an amino acid

sequence LEGGFYGP (SEQ ID NO: 74) or LEGGFYG (SEQ ID NO: 85). The peptides provided herein may have an amino acid sequence selected from those presented in Table 2 or 3 below for the Eagan strain of *H. influenzae* type b and corresponding amino acid sequences for other strains of *H. influenzae*.

In accordance with another aspect of the invention, an immunogenic composition is provided which comprises at least one active component selected from at least one nucleic acid molecule as provided herein, at least one recombinant protein as provided herein, at least one of the purified and isolated Tbp1 or Tbp2 proteins, as provided herein, at least one synthetic peptide, as provided herein, at least one purified and isolated truncated Tbp2 protein, as provided herein and a live vector, as provided herein, and a pharmaceutically acceptable carrier therefor or vector therefor. The at least one active component produces an immune response when administered to a host.

The immunogenic compositions provided herein may be formulated as a vaccine for *in vivo* administration to protect against diseases caused by bacterial pathogens that produce transferrin receptors. For such purpose, the compositions may be formulated as a microparticle, capsule or liposome preparation. Alternatively, the compositions may be provided in combination with a targeting molecule for delivery to specific cells of the immune system or to mucosal surfaces. The immunogenic composition may comprise a plurality of active components to provide protection against disease caused by a plurality of species of transferrin receptor producing bacteria. The immunogenic compositions may further comprise an adjuvant.

In accordance with another aspect of the invention, there is provided a method for inducing protection against infection or disease caused by *Haemophilus* or

other bacteria that produce transferrin receptor protein, comprising the step of administering to a susceptible host, such as a human, an effective amount of the immunogenic composition as recited above.

In accordance with another aspect of the invention, an antiserum or antibody specific for the recombinant protein, the isolated and purified Tbp1 protein or Tbp2 protein, synthetic peptide or the immunogenic composition, is provided.

In a further aspect, there is provided a live vector for delivery of transferrin receptor to a host, comprising a vector containing the nucleic acid molecule as described above. The vector may be selected from *Salmonella*, BCG, adenovirus, poxvirus, vaccinia and poliovirus. The vector may specifically be poliovirus and the nucleic acid molecule may code for a fragment of transferrin receptor having an amino acid sequence of LEGGFYGP (SEQ ID NO: 74) or LEGGFYG (SEQ ID NO: 85). The present invention further includes a plasmid vector having the identifying characteristics of pT7TBP2A, pT7TBP2B, pT7TBP2C or pT7TBP2D (ATCC designation Nos. 75931, 75932, 75933, 75934).

An additional aspect of the invention provides a strain of *Haemophilus* that does not produce transferrin receptor protein. Such strain may comprise a gene encoding transferrin receptor which is functionally disabled, such as by insertional mutagenesis. The *Haemophilus* strain may be one that has been attenuated and the attenuated strain may comprise the vector for delivery of transferrin receptor.

As mentioned above, one aspect of the invention provides novel Tbp1 or Tbp2 protein of a strain of *Haemophilus*, preferably a strain of *Haemophilus influenzae*, which is isolated and purified and free from the other. A yet further aspect of the present invention provides a method for producing such proteins.

Accordingly, in this yet further aspect, the present invention provides a method of producing an isolated and purified Tbp1 or Tbp2 protein of a strain of *Haemophilus*, comprising the steps of (a) providing a recombinant host expressing, in inclusion bodies, Tbp1 or Tbp2 protein, but not both; (b) growing the host to provide a cell mass; (c) disrupting the cell mass to provide a cell lysate; (d) fractionating the cell lysate to provide a first supernatant and a first pellet, the first supernatant comprising substantially a large proportion of soluble host proteins; (e) separating the first supernatant from the first pellet; (f) selectively extracting the first pellet to remove substantially all soluble host proteins and host membrane proteins therefrom to provide a second supernatant and an extracted pellet containing the inclusion bodies; (g) separating the second supernatant from the extracted pellet; (h) solubilizing the extracted pellet to provide a solubilized extract; and (i) fractionating the solubilized extract to provide a Tbp1 or Tbp2 protein containing fraction.

The cell lysate may be fractionated to provide the first supernatant and first pellet may be effected by at least one detergent extraction.

The solubilized extract may be fractionated by gel filtration to provide the Tbp1 or Tbp2 protein containing fraction, which may be subsequently dialyzed to remove at least the detergent and provide a further purified solution of Tbp1 or Tbp2 protein.

BRIEF DESCRIPTION OF DRAWINGS

The present invention will be further understood from the following description with reference to the drawings, in which:

Figure 1A shows the restriction map of two plasmid clones (pBHT1 and pBHT2) of the transferrin receptor operon of *Haemophilus influenzae* type b strain DL63.

Figure 1B shows the restriction map of clones S-4368-3-3 and JB-901-5-3 containing TfR genes from *H. influenzae* type b strain Eagan.

Figure 1C shows the restriction map of clone DS-712-1-3 containing the transferrin receptor gene from *H. influenzae* type b strain MinnA.

Figure 1D shows the restriction map of clone JB-1042-7-6 containing the transferrin receptor gene from the non-typable *H. influenzae* strain PAK 12085.

Figure 2 illustrates the organization and restriction maps of the cloned Tbp1 and Tbp2 genes of identified strains and the genetic organization of the TfR operon with two genes (*tbp1* and *tbp2*) in tandem forming an operon under the transcriptional regulation of a single promoter and also depicts the 3.0 kb DNA fragment of pBHIT2 used to probe libraries for TfR genes from the *Haemophilus* strains.

~~Figure 3 shows the nucleotide sequences of the~~ transferrin receptor genes (SEQ ID NO: 1) and their deduced amino acid sequences (SEQ ID NO: 5 - Tbp1 and SEQ ID NO: 6 - Tbp2) from *H. influenzae* type b, strain DL63. The underlined amino acid sequences correspond to peptides of Tbp1 identified by amino acid sequencing. The putative signal sequences are indicated by double overlining and correspond to residues 1 to 17 for Tbp2 ~~and 1 to 23 for Tbp1~~.

~~Figure 4 shows the nucleotide sequences of the~~ transferrin receptor genes (SEQ ID NO: 2) and their deduced amino acid sequences (SEQ ID NO: 7 - Tbp1 and SEQ ID NO: 8 - Tbp2) from *H. influenzae* type *b* strain Eagan. Putative -35, -10 and ribosomal binding site sequences ~~are overlined~~.

~~Figure 5 shows the nucleotide sequences of the~~ transferrin receptor genes (SEQ ID NO: 3) and their deduced amino acid sequences (SEQ ID NO: 9 - Tbp1 and SEQ

ID NO: 10 - Tbp2) from *H. influenzae* type b strain MinnA. Putative -35, -10 and ribosomal binding site sequences are overlined.

Figure 6 shows the nucleotide sequences of the transferrin receptor genes (SEQ ID NO: 4) and their deduced amino acid sequences (SEQ ID NO. 11 - Tbp1 and SEQ ID NO. 12 - Tbp2) from the non-typable *H. influenzae* strain PAK 12085. Putative -35, -10 and ribosomal binding site sequences are overlined.

Figure 7 shows the nucleotide sequences of the transferrin receptor genes (SEQ ID NO: 105) and their deduced amino acid sequences (SEQ ID NO. 106 -Tbp1 and SEQ ID NO. 107 - Tbp2) from the non-typable *H. influenzae* strain SB33.

Figure 8 shows the nucleotide sequence of the Tbp2 gene (SEQ ID NO: 108) and the deduced amino acid sequence (SEQ ID NO: 109 - Tbp2) from non-typable strain *H. influenzae* strain SB12.

Figure 9 shows the nucleotide sequence of the Tbp2 gene (SEQ ID NO: 110) and the deduced amino acid sequence (SEQ ID NO: 111 - Tbp2) from non-typable strain *H. influenzae* strain SB29.

Figure 10 shows the nucleotide sequence of the Tbp2 gene (SEQ ID NO: 112) and the deduced amino acid sequence (SEQ ID NO: 113 - Tbp2) from non-typable strain *H. influenzae* strain SB30.

Figure 11 shows the nucleotide sequence of the Tbp2 gene (SEQ ID NO: 114) and the deduced amino acid sequence (SEQ ID NO: 115 - Tbp2) from non-typable strain *H. influenzae* strain SB32.

Figure 12A shows the nucleotide sequences of the promoter regions and 5'-end of the *tbp2* genes from *H. influenzae* strains Eagan (SEQ ID NO: 116), MinnA (SEQ ID NO: 117), PAK 12085 (SEQ ID NO: 118) and SB33 (SEQ ID NO: 119). The coding strand primer used to amplify *tbp2* genes by PCR is underlined (SEQ ID NO: 120).

Figure 12B shows the nucleotide sequence of the intergenic region and 5'-end of the *tbp1* genes from *H. influenzae* strains Eagan (SEQ ID NO: 121), MinnA (SEQ ID NO: 122), DL63 (SEQ ID NO: 123), PAK 12085 (SEQ ID NO: 124), SB12 (SEQ ID NO: 125), SB29 (SEQ ID NO: 126), SB30 (SEQ ID NO: 127), and SB32 (SEQ ID NO: 128). The non-coding strand primer used to amplify the *tbp2* genes by PCR is underlined (SEQ ID NO: 129).

Figure 13 shows the agarose gel analysis of PCR amplified *tbp2* genes from non-typable *H. influenzae* strains SB12, SB29, SB30, SB32 and SB33. Lane 1 is SB33, lane 2 is SB12, lane 3 is SB29, lane 4 is SB30, lane 5 is SB32.

Figure 14 shows a comparison of the amino acid sequences of Tbp1 from *H. influenzae* strains Eagan, DL63, PAK 12085 and SB33 (SEQ ID NOS: 7, 5, 11 and 106), *N. meningitidis* strains B16B6 and M982 (SEQ ID NOS: 94 and 95), and *N. gonorrhoeae* strain FA19 (SEQ ID NO: 96).

Figure 15 shows a comparison of the amino acid sequence of Tbp2 from *H. influenzae* strains Eagan, DL63, PAK 12085, SB12, SB29, SB30 and SB32 (SEQ ID NOS: 8, 6, 12, 109, 110, 112, 114), *N. meningitidis* strains B16B6 and M982 (SEQ ID NOS: 97 and 98), *N. gonorrhoeae* strain FA19, and *Actinobacillus pleuropneumoniae* strains AP205 and AP37 (SEQ ID NOS: 99 and 100).

Figure 16A shows the predicted secondary structure of *H. influenzae* Tbp1 protein and Figure 16B shows the predicted secondary structure of *H. influenzae* Tbp2 protein.

Figure 17 shows the construction scheme of plasmid JB-1468-29 which expresses *H. influenzae* type b Eagan Tbp1 from *E. coli*.

Figure 18 shows the construction scheme of plasmid JB-1424-2-8 which expresses *H. influenzae* type b Eagan Tbp2 from *E. coli*.

Figure 19 shows the oligonucleotide pairs (SEQ ID NOS: 130, 131) used to construct plasmid JB-1424-2-8.

~~Figure 20 shows the sequence of oligonucleotide~~ pairs A (SEQ ID NOS: 86, 87), B (SEQ ID NOS: 88, 89), C (SEQ ID NOS: 90, 91) and D (SEQ ID NOS: 92, 93) for constructing Tbp1 and Tbp2 expression plasmids.

Figure 21 shows the construction scheme of plasmid JB-1600-1 which expresses *H. influenzae* strain SB12 Tbp2 ~~from *E. coli*~~.

Figure 22 shows SDS-PAGE gels of products from the expression of *Haemophilus* type b Eagan Tbp1 protein, Eagan Tbp2 protein, and non-typable *H. influenzaea* SB12 Tbp2 protein from *E. coli*. Lane 1, JB-1476-2-1 (T7/Eagan Tbp1) at t_o; lane 2, JB-1476-2-1 at t=4h induction; lane 3, molecular weight markers of 200 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa and 31 kDa; lane 4, JB-1437-4-1 (T7/Eagan Tbp2) at t_o; lane 5, JB-1437-4-1 at t=4h induction; lane 6, JB-1607-1-1 (T7/SB12 Tbp2) at t_o; lane 7, JB-1607-1-1 at t=4h induction.

Figure 23 shows a purification scheme for recombinant Tbp1 and Tbp2 expressed from *E. coli*.

Figure 24 shows an analysis of the purity of recombinant Tbp1 and Tbp2 purified by the scheme of Figure 23. Lane 1 contains molecular weight size markers (106, 80, 49.5, 32.5, 27.5 and 18.5 kDa), Lane 2 is *E. Coli* whole cell lysate. Lane 3 is solubilized inclusion bodies. Lane 4 is purified Tbp1 or Tbp2.

Figure 25 shows the immunogenicity of rTbp1 (upper panel) and rTbp2 (lower panel) in mice.

Figure 26 shows the reactivity of anti-Eagan rTbp1 antisera with various *H. influenzae* strains on a Western blot. Lane 1, BL21/DE3; lane 2, SB12-EDDA; lane 3, SB12 +EDDA; lane 4, SB29 - EDDA; lane 5, SB29 +EDDA; lane 6, SB33 -EDDA; lane 7, SB33 + EDDA; lane 8, Eagan -EDDA; lane 9, Eagan +EDDA; lane 10, *B. catarrhalis* 4223 - EDDA; lane 11, *B. catarrhalis* 4223 +EDDA; lane 12, *N.*

meningitidis 608 - EDDA; lane 13, *N. meningitidis* 608 + EDDA; lane 14, induced JB-1476-2-1 expressing recombinant Eagan Tbp1; lane 15, molecular weight markers. Specific ~ 95 kDa bands reacted with the anti-Tbp1 antisera in lanes 3, 4, 5, 7, 8 and 9, corresponding to *H. influenzae* strains SB12, SB29, SB33 and Eagan; ~ 110 kDa bands in lanes 10 and 11, corresponding to *B. catarrhalis* strain 4223; and ~ 80 kDa bands in lanes 12 and 13, corresponding to *N. meningitidis* 608.

Figure 27 shows the reactivity of anti-Eagan rTbp2 antisera with various *H. influenzae* strains on a Western blots. Lane 1, molecular weight markers; lane 2, induced JB-1437-4-1 expressing recombinant Eagan Tbp2; lane 3, SB12-EDDA; lane 4, SB12 +EDDA; lane 5, SB29 -EDDA; lane 6, SB29 +EDDA; lane 7, SB30 -EDDA; lane 8, SB30 +EDDA; lane 9, SB32 -EDDA; lane 10, SB33-EDDA; lane 11, SB33 +EDDA; lane 12, PAK -EDDA; lane 13, PAK +EDDA; lane 14, Eagan -EDDA; lane 15, Eagan +EDDA. Specific bands of 60-70 kDa were reactive with the anti-Tbp2 antisera in lanes, 3, 6, 7, 8, 13, 14 and 15, i.e. strains SB12, SB29, SB30, PAK and Eagan.

Figure 28 shows the construction of plasmids pUHIT1KFH and pUHIT1KFP used to generate strains of *H. influenzae* that do not produce transferrin receptor.

Figure 29 shows the construction of plasmids encoding chimeric polioviruses expressing an epitope derived from transferrin receptor protein that is conserved among bacteria that produce transferrin receptor protein.

Figure 30 is a Western blot showing the reactivity of antisera produced by immunization of rabbits with poliovirus chimeras expressing an epitope derived from transferrin receptor protein that is conserved among bacteria that produce transferrin receptor protein. Panel A shows a Coomassie Brilliant Blue-stained gel showing purified recombinant Tbp2 from *H. influenzae*

strain SB12 expressed in *E. coli* (lane 1), purified Tbp2 from *Branhamella catarrhalis* strain 4223 (lane 2), a whole cell lysate of iron-limited *B. catarrhalis* strain 4223 (lane 3), a whole cell lysate of *E. coli* JM109 grown under non-iron limited conditions (lane 5). Panel B shows results of a Western blot of a replicate gel using a pool of the sera collected on day 27 from rabbits immunised with PV1TBP2A (rabbits 40, 41 and 42). Panel C shows the results for a pool of prebleed sera from the same, which displayed minimal specific reactivity.

~~Figure 31 illustrates a number of truncated analogues of transferrin receptor protein Tbp2.~~

~~Figure 32 shows the binding of truncated Tbp2 proteins to transferrin.~~

In some of the above Figures, the following abbreviations have been used to designate particular site specific restriction endonucleases: R, *Eco* RI; Ps, *Pst* I; H, *Hind* III; Bg, *Bgl* II; Nde, *Nde* I; Ear, *Ear* I; and Sau, *Sau*3A I.

In Figure 28, the following abbreviations have been used to designate particular site specific restriction endonucleases: A, *Acc* I; B *Bam* HI; E, *Eco* RI; O, *Xho* I; H, *Hind* III; Ps, *Pst* I; V, *Eco* RV; X, *Xba* I, G, *Bgl* II; S, *Sal* I; K, *Kpn* I; and S*, *Sac* I.

GENERAL DESCRIPTION OF THE INVENTION

Any *Haemophilus* strain may be conveniently used to provide the purified and isolated nucleic acid which may be in the form of DNA molecules, comprising at least a portion of the nucleic acid coding for a transferrin receptor as typified by embodiments of the present invention. Such strains are generally available from clinical sources and from bacterial culture collections, such as the American Type Culture Collection.

According to an aspect of the invention, the transferrin receptor protein may be isolated from

Haemophilus strains by the methods described by Schryvers (1989), Ogunnaviwo and Schryvers (1992) and US patent 5,141,743, the subject matter of which is hereby incorporated by reference. Although the details of an appropriate process are provided in patent US 5,141,743, a brief summary of such process is as follows. Isolation of transferrin receptor is achieved by isolating a membrane fraction from a bacterial strain expressing transferrin binding activity and purifying the transferrin receptor by an affinity method involving the sequential steps of prebinding of transferrin to the transferrin receptor in the membrane fraction, solubilising the membrane, immobilising the transferrin and separating the transferrin receptor from the immobilised transferrin. Alternatively, the receptor proteins may be isolated by a modification of the above method in which the prebinding step is avoided and a high concentration of salt is included in the solubilization buffer to allow direct isolation with immobilized transferrin as described in Ogunnariwo and Schryvers (1992).

In this application, the term "transferrin receptor" is used to define a family of Tbp1 and/or Tbp2 proteins which includes those having variations in their amino acid sequences including those naturally occurring in various strains of, for example, *Haemophilus*. Other bacterial sources of transferrin receptor include, but are not limited to, species of *Neisseria*, *Branhamella*, *Pasteurella* and *Actinobacillus*. Some, if not all, of these bacteria contain both Tbp1 and Tbp2. The purified and isolated DNA molecules comprising at least a portion coding for transferrin receptor of the present invention also include those encoding functional analogs of transferrin receptor. In this application, a first protein or peptide is a "functional analog" of a second protein if the first protein is immunologically related

to and/or has the same function as the second protein or peptide. The functional analog may be, for example, a fragment of the protein, such as those shown in Figure 31, or a substitution, addition or deletion mutant thereof.

In one particular embodiment, the transferrin receptor was isolated from *H. influenzae* type b strain DL63 and purified by affinity chromatography methods, as described by Schryvers (1989), Ogunnariwo and Schryvers (1992) and in US patent 5,141,743. The isolated and purified transferrin receptor was used to generate anti-TfR antisera in rabbits. Chromosomal DNA from *H. influenzae* type b strain DL63 was mechanically sheared, *Eco*RI linkers added, and a λZAP expression library constructed. The library was screened with the anti-TfR rabbit antisera and two positive clones (pBHIT1 and pBHIT2) were obtained which had overlapping restriction maps (Figure 1A and Figure 2). The clones were sequenced and two large open reading frames were identified (Figure 2). The nucleotide sequences of the transferrin receptor genes Tbp1 and Tbp2 (SEQ ID NO: 1) from *H. influenzae* DL63 and their deduced amino acid sequences (SEQ ID NO: 5 - Tbp1 and SEQ ID NO: 6 - Tbp2) are shown in Figure 3. The sequence analysis showed the TfR operon to consist of two genes (Tbp1 and Tbp2) arranged in tandem and transcribed from a single promoter (as particularly shown in Figure 2 and Figure 3). The Tbp2 protein tends to vary in molecular weight depending on the species whereas the Tbp1 protein tends to have a more consistent molecular weight with some variability across the various bacteria which have TfR genes. The molecular weight of Tbp1 is usually in the range of 94 to 106,000 whereas the molecular weight of Tbp2 varies considerably from 58 to 98 000.

Amino acid sequencing of the N-termini and cyanogen bromide fragments of transferrin receptor from *H.*

influenzae DL63 was performed. The N-terminus of Tbp2 was blocked but amino acid sequences were identified by sequencing of Tbp1 and are indicated by underlining within the protein sequence of Figure 3. These peptide sequences are **Glu Thr Gln Ser Ile Lys Asp Thr Lys Glu Ala Ile Ser Ser Glu Val Asp Thr** (as shown in Figure 3, SEQ ID NO: 101) and **Leu Gln Leu Asn Leu Glu Lys Lys Ile Gln Gln Asn Trp Leu Thr His Gln Ile Ala Phe** (as shown in Figure 3; SEQ ID NO: 102). The signal sequence of Tbp1 and the putative signal sequence of Tbp2 are indicated by double overligning in Figure 3. The putative signal sequence for Tbp1 is **Met Thr Lys Lys Pro Tyr Phe Arg Leu Ser Ile Ile Ser Cys Leu Leu Ile Ser Cys Tyr Val Lys Ala** (SEQ ID NO: 103). The putative signal sequence for Tbp2 is **Met Lys Ser Val Pro Leu Ile Ser Gly Gly Leu Ser Phe Leu Leu Ser Ala** (SEQ ID NO: 104). The derived amino acid sequence of the N-terminal region of Tbp2 indicates that it is a lipoprotein.

Chromosomal DNA from *H. influenzae* type b strain Eagan was prepared and libraries were generated. The first library was constructed from DNA partially digested with *Sau*3A I, size-fractionated for ~5-10 kb fragments, and cloned into a pUC-based plasmid. The second library was constructed from *Eco* RI- restricted chromosomal DNA fragments cloned into λZAP. Both libraries were probed with a 5'-fragment of the pBHIT clone as shown in Figure 2 and partial clones of the TfR genes of *H. influenzae* Eagan termed S-4368-3-3 and JB-901-5-3 were obtained. Thus, referring to Figures 1B and 2, there is illustrated according to further aspects of the present invention, plasmid clones S-4368-3-3 and JB-901-5-3 encoding Tbp1 and Tbp2 from *H. influenzae* type b strain Eagan. The DNA sequences of the Tbp1 and Tbp2 genes (SEQ ID NO: 2) from *H. influenzae* type b strain Eagan and their deduced amino acid sequences (SEQ ID NOS: 7 and 8) are shown in Figure 4 with the Tbp2 sequence being the first gene in the

operon. In Figure 4, putative -35, -10 and ribosomal binding site sequences are overlined.

Chromosomal DNA from *H. influenzae* type b strain MinnA was prepared and the DNA partially digested with *Sau*3A I, size-fractionated for 10-20 kb fragments, and cloned into the *Bam*HI site of EMBL3. The library was probed with the 5'-fragment of the pBHIT clone (Figure 2) and a full-length clone encoding TfR (DS-712-1-3) was obtained. Referring to Figures 1C and 2, there is illustrated according to additional aspects of the present invention, plasmid clone DS 712-1-3 encoding Tbp1 and Tbp2 from *H. influenzae* type b strain MinnA. The DNA sequences of Tbp1 and Tbp2 (SEQ ID NO: 3) and their deduced amino acid sequences (SEQ ID NO: 9 - Tbp1 and SEQ ID NO: 10 - Tbp2) from *H. influenzae* type b strain MinnA are shown in Figure 5 where the Tbp2 sequence is first in the operon. In Figure 5, Putative -35, -10 and ribosomal binding site sequences are overlined.

Chromosomal DNA from the non-typable *H. influenzae* strain PAK 12085 was prepared. The DNA was partially digested with *Sau*3A I, size-fractionated for 10-20 kb fragments, and cloned into the BamH I site of EMBL3. The library was probed with the fragments of the pBHIT clone (Figure 2) and a full-length clone encoding TfR (JB-1042-7-6) was obtained. The restriction map of clone JB-1042-7-6 is shown in Figures 1D and 2 and the nucleotide sequences of the Tbp1 and Tbp2 genes (SEQ ID NO: 4) from *H. influenzae* PAK 12085 and their deduced amino acid sequences are shown in Figure 6 (SEQ ID NOS: 11, 12), with the Tbp2 sequence first. In Figure 6, Putative -35, -10 and ribosomal binding site sequences are overlined.

Chromosomal DNA from the otitis-media derived non-typable *H. influenzae* strain SB33 was prepared. The DNA was partially digested with *Sau*3A I, size-fractionated for 10-20 kb fragments, and cloned into the BamH I site of EMBL3. The library was probed with the fragments of

the pBHIT clone (Figure 2) and a full-length clone encoding TfR (JB-1031-2-9) was obtained. The restriction map of clone JB-1031-2-9 is shown in Figure 2 and the nucleotide sequences of the Tbp1 and Tbp2 genes (SEQ ID NO: 105) from *H. influenzae* SB33 and their deduced amino acid sequences are shown in Figure 7 (SEQ ID NOS: 106, 107), with the Tbp2 sequence first. The SB33 *tbp2* gene was found to have a single base deletion which resulted in a frame-shift at residue 126 and premature truncation of the resulting protein at residue 168.

PCR amplification of the *tbp2* genes from otitis media-derived NTHi strains SB12, SB29, SB30 and SB32 was performed and the genes sequenced.

The nucleotide sequence of the *tbp2* genes from non-typable *H. influenzae* strains SB12 (SEQ ID NO: 105), SB29 (SEQ ID NO: 108), SB30 (SEQ ID NO: 110) and SB32 (SEQ ID NO: 112) are shown in Figures 8, 9, 10 and 11 respectively.

All of the amplified *tbp2* genes were found to encode full-length Tbp2 proteins indicating that the defective *tbp2* gene of strain SB33 was atypical.

The three *H. influenzae b* strains all had identical short intergenic sequences of only 13 bp between *tbp2* and *tbp1*, but the NTHi strains PAK 12085 and SB33 had longer intergenic sequences of 27 bp (Figure 12).

Strain SB12 had a 13 bp intergenic sequence identical to that found in the *H. influenzae b* strains while strains SB29, SB30 and SB32 contained longer intergenic sequences (27-30 bp) as found in the other NTHi strains PAK 12085 and SB33 (Figure 2B). All nine strains have a common core conserved 13 bp sequence between their *tbp2* and *tbp1* genes.

A pentapeptide sequence near the amino terminus of *H. influenzae* Tbp1 was identified (Figure 12) which is similar to the TonB box. The *tonB* gene of *H. influenzae*

has been recently cloned and sequenced (Jarosik et al., 1994).

The amino acid sequences of Tbp1 from *H. influenzae* strains Eagan/MinnA, DL63, PAK 12085 and SB33 strains are compared in Figure 14. The Tbp1 proteins of Eagan and MinnA are identical and 912 amino acids in length, that of DL63 has 914 residues, that of PAK 12085 has 914 residues, and that of SB33 has 911 residues. The *H. influenzae* Tbp1 proteins are highly conserved with 95-100% sequence identity. The amino acid sequences of Tbp2 from *H. influenzae* strains Eagan/MinnA, DL63, PAK 12085 SB12, SB29, SB30 and SB32 are compared in Figure 15. The Tbp2 proteins of Eagan and MinnA are identical and contain 660 amino acids, that of DL63 has 644 residues, and that of PAK 12085 has 654 residues. There is a single base deletion in the SB33 *tbp2* gene which results in a frame-shift at residue 126 and premature trunction of the resulting protein at residue 168. The missing base was confirmed by direct sequencing of PCR amplified chromosomal DNA. With the exception of Eagan and MinnA which are identical, the Tbp2 protein sequences are less conserved with only 66-70% identity, but there are several short segments of conserved sequence which can be identified in Figure 15. The PCR amplified *tbp2* genes from strains SB12, SB29, SB30 and SB32 were all found to encode full-length Tbp2 proteins. There was sequence and size heterogeneity amongst the deduced Tbp2 proteins wherein SB12 had 648 amino acids, SB29 had 631 residues, SB30 had 630 residues and SB32 had 631 residues.

Putative secondary structures of Eagan Tbp1 and Tbp2 were determined (Figures 16A and 16B). Both proteins have several transmembrane domains, with Tbp1 traversing the membrane 20 times and Tbp2 crossing it 12 times. Three exposed conserved epitopes were identified in the Tbp1 amino-terminal region (DNEVTGLGK - SEQ ID NO: 43, EQVLN/DIRDLTRYD - SEQ ID NOS: 139 and 140, and

GAINEIEYENVKAVEISK - SEQ ID NO: 141) and one in the C-terminal region (GI/VYNLF/LNYRYVTWE - SEQ ID NOS: 142 and 143). Only three small conserved regions can be identified within the Tbp2 proteins of the human pathogens: CS/LGGG(G)SFD - SEQ ID NOS: 75, 144 and 145 at the N-terminal, LE/SGGFY/FGP - SEQ ID NOS: 74 and 146 located internally, and VVFGAR/K - SEQ ID NOS: 83 and 84 at the C-terminus

The discovery that the Tbp2 amino acid sequence varies between strains of *Haemophilus* allows for the grouping of *Haemophilus* into sub-groups defined by the same Tbp2 amino acid sequence. This discovery allows the rational selection of a minimal number of Tbp1 and/or Tbp2 sequences or synthetic peptides representing epitopes shared by such subtypes within strains of *Haemophilus* to be used in immunogenic compositions for, for example, immunization against the diseases caused by *Haemophilus* and other bacteria that produce transferrin receptor with sequence similarities to Tbp1 and Tbp2 from *Haemophilus* species. Thus, a minimal number of transferrin receptor, analogs, fragments, and/or peptides, may be used to immunize against many or all strains of *Haemophilus* and other bacterial pathogens that produce transferrin receptor.

Furthermore, the amino acid sequences of the transferrin receptor from a range of bacterial pathogens (*H. influenzae* type b, non-typable *H. influenzae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *Actinobacillus* (*Haemophilus*) *pleuropneumoniae*) were compared as shown in Figures 14 and 15. This analysis revealed regions of Tbp1 and Tbp2 which are conserved between all of these bacteria. Some of such conserved sequences are contained in peptides in Tables 2 and 3. In particular the sequences DNEVTGLGK (SEQ ID: 43), EQVLNIRDLTRYDPGI (SEQ ID NO: 44), EQVLNIRDLTRYDPGISVVEQG RGASSGYSIRGMD (SEQ ID NO: 45), GAINEIEYENVKAVEISKG (SEQ

ID NO: 46) and GALAGSV (SEQ ID NO: 47) are conserved in Tbp1 (Table 1 and Figure 14). Particular conserved sequences in Tbp2 include LEGGFYGP (SEQ ID NO: 74), CSGGGSFD (SEQ ID NO: 75), YVYSGL (SEQ ID NO: 76), CCSNLSYVKFG (SEQ ID NO: 77), FLLGHRT (SEQ ID NO: 78), EFNVOF (SEQ ID NO: 79), NAFTGTA (SEQ ID NO: 80), VNGAFYG (SEQ ID NO: 81), ELGGYF (SEQ ID NO: 82), VVFGAR (SEQ ID NO: 83) and VVFGAK (SEQ ID NO: 84) (Table 2 and Figure 15).

The discovery of conserved sequences within the transferrin receptor of a range of bacterial pathogens allows the selection of a minimal number of antigens having particular amino acid sequences (including in the form of synthetic peptides) to immunize against the disease caused by pathogens that have transferrin receptors. Such bacteria in addition to those recited above include other species of *Neisseria*, such as *Neisseria gonorrhoeae*, and *Branhamella*, including *Branhamella catarrhalis*. Such conserved amino acid sequences among many bacterial pathogens permits the generation of TfR specific antibodies, including monoclonal antibodies, that recognize most if not all transferrin receptors. Antiserum was raised against peptides corresponding to conserved portions of the transferrin receptor. This antiserum recognized the transferrin receptor in *Branhamella catarrhalis*. Such antisera are useful for the detection and neutralization of most if not all bacteria that produce TfR protein and are also useful for passive immunization against the diseases caused by such pathogens. Diagnostic assays and kits using such conserved amino acid sequences are useful to detect many if not all bacteria that produce transferrin receptor.

Epitopes containing the afore-mentioned amino acid sequences can be delivered to cells of the immune system by the use of synthetic peptides containing such

sequences, or by the use of live vectors expressing such sequences, or by the direct administration of nucleic acid molecules encoding the amino acid sequence.

Some peptides containing conserved amino acid sequences within the Tbp1 proteins of *H. influenzae* type b strains Eagan, MinnA, DL63 and the nontypable strain PAK 12085 are shown in Table 2. Antibodies to some of these peptides were raised in guinea pigs (Table 4). Peptides containing conserved amino acid sequences within the Tbp2 proteins of *H. influenzae* type b strains Eagan, Minn A, DL63 and the nontypable strain PAK 12085 are shown in Table 3. Antibodies to some of these peptides were raised in guinea pigs (Table 4).

The coding sequences of the Tbp1 and Tbp2 genes may be cloned into appropriate expression vectors to produce recombinant proteins. Recombinant Tbp1 and Tbp2 were expressed from *E. coli* using the T7 expression system. The *tbp1* gene encoding the mature Eagan Tbp1 protein was cloned in-frame behind the T7 promoter generating plasmid JB-1468-29, as shown in Figure 17. When introduced into BL21/DE3 cells and induced with IPTG or lactose, Eagan Tbp1 protein was expressed as shown in Figure 22.

The *tbp2* gene encoding the mature Tbp2 protein was cloned in-frame behind the T7 promotor generating plasmid JB-1424-2-8 as shown in Figure 18. When introduced into *E. coli* cells and induced as above, Tbp2 protein was expressed as shown in Figure 22.

The *tbp2* gene from strain NTHi SB12 was amplified by PCR. The resultant amplified DNA contains the authentic *H. influenzae* Tbp2 signal sequence before the mature protein. The SB12 *tbp2* gene encoding the signal sequence and the mature protein was cloned into the pT7-7 expression system as shown in Figure 21. When the resultant plasmid (JB-1600-1) was introduced into *E. coli* BL21/DE3 cells and induced, SB12 Tbp2 was expressed, as shown in Figure 22.

Recombinant proteins Tbp1 and Tbp2 produced in *E. coli* as inclusion bodies were purified by the scheme shown in Figure 23. The purified proteins were at least about 70% pure as shown in Figure 24. Immunogenicity studies were performed in mice with the purified recombinant Tbp1 and Tbp2 proteins. Both proteins elicited a good immune response in mice at 3-10 μg doses (Figure 25).

Antisera raised to recombinant Tbp1 or Tbp2 derived from one *H. influenzae* strain are cross-reactive with other strains, making these potentially useful diagnostic reagents (Figures 26 and 27).

Plasmids pUHIT1KFH and pUHITKFP shown in Figure 28, contain a selectable antibiotic resistance marker cloned within the transferrin receptor operon and were constructed to insertionally inactivate the transferrin receptor operon. These plasmids were used to transform *Haemophilus* to generate strains that do not produce transferrin receptor Tbp1 and/or Tbp2 as described in Example 19. Such strains are useful as negative controls (since they do not produce TfR) in *in vitro* and *in vivo* detection and diagnostic embodiments. Such strains are also expected to be attenuated for *in vivo* growth and are useful as live vaccines to provide protection against diseases caused by *Haemophilus*.

As discussed above, epitopes of transferrin receptor proteins can be delivered to cells of the immune system by the use of live vectors expressing such amino acid sequences and the live vector may be poliovirus. Referring to Figure 29 there is illustrated the construction of hybrid polioviruses expressing an epitope of transferrin receptor protein including the conserved epitope from Tbp2 LEGGFYGP (SEQ ID NO: 74). Such viruses were recognized by antibodies raised against a peptide incorporating the amino acid sequence LEGGFYGP (SEQ ID NO: 74) (Table 5) indicating that the viruses expressed

this sequence in an antigenically recognisable form. PV1TBP2A and PV1TBP2B were also neutralized by rabbit antisera raised against *H. influenzae* strain DL63 *tbp2*, indicating that at least these two viruses expressed the sequence in a form recognisable to antibodies raised against the protein. All viruses were neutralisable by anti-PV1 sera, indicating that the changes in polio neutralization antigenic site I had not significantly affected other antigenic sites on the viruses. Furthermore, rabbit antiserum produced by immunization with poliovirus chimera PV1TBP2A or PV1TBP2B recognized a peptide incorporating the amino acid sequence LEGGFYGP (SEQ ID NO: 74). This indicates that the sequences expressed by PV1TB2A and PV1TBP2B are immunogenic and elicit antibodies capable of recognizing the same sequence in the context of a synthetic peptide.

Referring to Figure 30, panel A shows an SDS PAGE gel showing purified recombinant *tbp2* from *H. influenzae* strain SB12 expressed in *E. coli* (lane 1), *tbp2* from *Branhamella catarrhalis* strain 4223 (lane 2), a whole cell lysate of iron-limited *B. catarrhalis* strain 4223 (land 3), a whole cell lysate of iron-limited *E. coli* JM109 (lane 4), and a whole cell lysate of *E. coli* JM109 grown under non-iron limited conditions (lane 5). Panel B shows results of a Western blot of a replicate gel using a pool of sera from rabbits immunized with PV1TBP2A. There was a strong reaction with the purified transferrin-binding proteins in lanes 1 and 2, and with a similar sized band in lane 3. There was no significant reaction with any *E. coli* proteins (lanes 4 and 5). Panel C shows the results for a pool of prebleed sera from the same rabbits, which displayed minimal specific reactivity. These results show that PV1TBP2A is able to induce antisera specific for transferrin binding proteins from *H. influenzae* and *B. catarrhalis*, and that the

antisera can distinguish *B. catarrhalis* from *E. coli*, which does not express an equivalent protein.

Guinea pig anti-Eagan rTbp1, anti-Eagan rTbp2, and anti-SB12 rTbp2 antisera were used to screen a panel of *H. influenzae* strains for antigenic conservation of the Tbp1 and Tbp2 proteins. Of 33 strains screened by Western blot with anti-Eagan rTbp1 antisera, all had a reactive band of ~100 kDa. Of 89 strains screened by Western blot with anti-Eagan rTbp2 antisera, 85 had a reactive band of 60-90 kDa. Of 86 strains screened by Western blot with anti-SB12 rTbp2 antisera, 82 had a reactive band of 60-90 kDa. Only one strain was not recognized by either anti-Eagan rTbp2 or anti-SB12 rTbp2 antisera, and that was NTHi strain SB33 which has a defective *tbp*B gene. These data indicate that transferrin receptor proteins are highly conserved in strains of *H. influenzae* and support the use of these proteins as antigens and in immunogenic compositions, including vaccines, for immunization against disease cause by *H. influenzae* and diagnosis thereof.

The infant rat model of bacteremia (Loeb et al, 1987) was used to assess the protective abililty of anti-Eagan rTbp1 and anti-Eagan rTbp2 antisera. Anti-Eagan rTbp1 antisera raised in either rabbits or guinea pigs was not protective in this model but anti-Eagan rTbp2 antisera raised in rabbits or guinea pigs was protective (Table 7). These data indicate the use for rTbp2 proteins as protective antigens against disease caused by *H. influenzae*.

The chinchilla model of otitis media (Barenkamp et al, 1986) was used to assess the protective ability of SB12 rTbp2. Data indicated that compared with the control group, the immunized animals had less severe disease.

~~. In further embodiments, there is provided a number of truncated analogues at transferrin receptor protein Tbp2 as shown in Table 8 and Fiure 31 below, and nucleic~~

acid molecules encoding the same. Some of such truncated analogues are highly expressed in recombinant expression systems (such as *E.coli*) and represent appropriate antisera and immunogens in diagnostic and vaccination embodiments of the invention.

The purified and isolated DNA molecules comprising at least a portion coding for a transferrin receptor of a species of *Haemophilus* typified by the embodiments described herein are advantageous as:

- nucleic acid probes for the specific identification of *Haemophilus* strains *in vitro* or *in vivo*.

- the products encoded by the DNA molecules are useful as diagnostic reagents, antigens for the production of *Haemophilus*-specific antisera, for vaccination against the diseases caused by species of *Haemophilus* and (for example) detecting infection by *Haemophilus*.

- peptides corresponding to portions of the transferrin receptor as typified by the embodiments described herein are advantageous as diagnostic reagents, antigens for the production of *Haemophilus*-specific antisera, for vaccination against the diseases caused by species of *Haemophilus* and (for example) for detecting infection by *Haemophilus*.

The transferrin receptor encoded by the nucleic acid molecules of the present invention, fragments and analogs thereof, and peptides containing sequences corresponding to portions of the transferrin receptor that are conserved between various isolates of *Haemophilus* and other bacteria that produce transferrin receptor, are useful in diagnosis of and immunization against diseases caused by any bacterial strain that produces transferrin receptor. In particular, peptides containing the sequences LEGGFYGP are conserved in the transferrin receptor proteins of many bacterial pathogens that

produce transferrin receptor and are appropriate for diagnosis of and immunization against diseases caused by bacteria that produce transferrin receptor. Such bacteria include but are not limited to species of *Haemophilus, Neisseria* (including *N. meningitidis* and *N. gonorrhoeae*) and *Branhamella* (including *B. catarrhalis*).

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of vaccination, diagnosis, treatment of, for example, *Haemophilus* infections, and infections with other bacterial pathogens that produce transferrin receptor and the generation of immunological reagents. A further non-limiting discussion of such uses is further presented below.

1. Vaccine Preparation and Use

Immunogenic compositions, suitable to be used as vaccines, may be prepared from immunogenic transferrin receptor, analogs and fragments thereof and/or peptides as disclosed herein. The vaccine elicits an immune response which produces antibodies, including anti-transferrin receptor antibodies and antibodies that are opsonizing or bactericidal. Should the vaccinated subject be challenged by *Haemophilus* or other bacteria that produce a transferrin receptor, the antibodies bind to the transferrin receptor and thereby prevent access of the bacteria to an iron source which is required for viability. Furthermore, opsonizing or bactericidal anti-TfR antibodies may also provide protection by alternative mechanisms.

Vaccines containing peptides are generally well known in the art, as exemplified by U.S. Patents 4,601,903; 4,599,231; 4,599,230; and 4,596,792; all of which references are incorporated herein by reference. Immunogenic compositions including vaccines may be prepared as injectables, as liquid solutions or emulsions. The transferrin receptor, analogs and

fragments thereof and/or peptides may be mixed with pharmaceutically acceptable excipients which are compatible with the transferrin receptor, fragments analogs or peptides. Such excipients may include, water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions and vaccines may further contain auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness of the vaccines. Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously or intramuscularly. Alternatively, the immunogenic compositions formed according to the present invention, may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the immunogenic composition may be administered to mucosal surfaces by, for example, the nasal or oral (intragastric) routes. The immunogenic composition may be provided in combination with a targeting molecule for delivery to specific cells of the immune system or to mucosal surfaces. Some such targeting molecules include strain B12 and fragments of bacterial toxins, as described in WO 92/17167 (Biotech Australia Pty. Ltd.), and monoclonal antibodies, as described in U.S. Patent No. 5,194,254 (Barber et al). Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkalene glycols or triglycerides. Oral formulations may include normally employed incipients such as, for example, pharmaceutical grades of saccharine, cellulose and magnesium carbonate. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10-95% of the transferrin receptor, fragment analogs and/or peptides.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize antibodies, and if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms of the transferrin receptor, analogs and fragments thereof and/or peptides. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage of the vaccine may also depend on the route of administration and will vary according to the size of the host.

The nucleic acid molecules encoding the transferrin receptor of the present invention may also be used directly for immunization by administration of the DNA directly, for example by injection for genetic immunization or by constructing a live vector such as *Salmonella*, BCG, adenovirus, poxvirus, vaccinia or poliovirus. A discussion of some live vectors that have been used to carry heterologous antigens to the immune system are discussed in for example O'Hagan (1992). Processes for the direct injection of DNA into test subjects for genetic immunization are described in, for example, Ulmer *et al.*, 1993.

The use of peptides *in vivo* may first require their chemical modification since the peptides themselves may not have a sufficiently long serum and/or tissue half-life and/or sufficient immunogenicity. Such chemically modified peptides are referred to herein as "peptide

analogs". The term "peptide analog" extends to any functional chemical equivalent of a peptide characterized by its increased stability and/or efficacy and immunogenicity *in vivo* or *in vitro* in respect of the practice of the invention. The term "peptide analog" is also used herein to extend to any amino acid derivative of the peptides as described herein. Peptide analogs contemplated herein are produced by procedures that include, but are not limited to, modifications to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide synthesis and the use of cross-linkers and other methods which impose conformational constraint on the peptides or their analogs.

Examples of side chain modifications contemplated by the present invention include modification of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidation with methylacetimidate; acetylation with acetic anhydride; carbamylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6, trinitrobenzene sulfonic acid (TNBS); alkylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5′-phosphate followed by reduction with $NaBH_4$.

The guanidino group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2, 3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation via o-acylisourea formation followed by subsequent derivatisation, for example, to a corresponding amide.

Sulfhydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of

mixed disulphides with other thiol compounds; reaction with maleimide; maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulfonic acid, phenylmercury chloride, 2-chloromercuric-4-nitrophenol and other mercurials; carbamylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphonyl halides. Tryosine residues may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid-, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids.

Immunogenicity can be significantly improved if the antigens are co-administered with adjuvants, commonly used as an 0.05 to 1.0 percent solution in phosphate - buffered saline. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune responses.

Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines. Intrinsic adjuvants, such as lipopolysaccharides, normally are the components of the killed or attenuated bacteria used as vaccines. Extrinsic adjuvants are immunomodulators which are typically non-covalently linked to antigens and are formulated to enhance the host immune responses. Thus, adjuvants have been identified that enhance the immune response to antigens delivered parenterally. Some of these adjuvants are toxic, however, and can cause undesirable side-effects, making them unsuitable for use in humans and many animals. Indeed, only aluminum hydroxide and aluminim phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines. The efficacy of alum in increasing antibody responses to diptheria and tetanus toxoids is will established and, more recently, a HBsAg vaccine has been adjuvanted with alum. While the usefulness of alum is well established for some applications, it has limitations. For example, alum is ineffective for influenza vaccination and inconsistently elicits a cell mediated immune response. The antibodies elicited by alum-adjuvanted antigens are mainly of the IgG1 isotype in the mouse, which may not be optimal for protection by some vaccinal agents.

A wide range of extrinsic adjuvants can provoke potent immune responses to antigens. These include saponins complexed to membrane protein antigens (immune stimulating complexes), pluronic polymers with mineral oil, killed mycobacteria and mineral oil, Freund's complete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as lipid A, and liposomes.

To efficiently induce humoral immune responses (HIR) and cell-mediated immunity (CMI), immunogens are

emulsified in adjuvants. Many adjuvants are toxic, inducing granulomas, acute and chronic inflammations (Freund's complete adjuvant, FCA), cytolysis (saponins and pluronic polymers) and pyrogenicity, arthritis and anterior uveitis (LPS and MDP). Although FCA is an excellent adjuvant and widely used in research, it is not licensed for use in human or veterinary vaccines because of its toxicity.

Desirable characteristics of ideal adjuvants include:

(1) lack of toxicity;

(2) ability to stimulate a long-lasting immune response;

(3) simplicity of manufacture and stability in long-term storage;

(4) ability to elicit both CMI and HIR to antigens administered by various routes, if required;

(5) synergy with other adjuvants;

(6) capability of selectively interacting with populations of antigen presenting cells (APC);

(7) ability to specifically elicit appropriate T_H1 or T_H2 cell-specific immune responses; and

(8) ability to selectively increase appropriate antibody isotype levels (for example, IgA) against antigens.

US Patent No. 4,855,283 granted to Lockhoff et al on August 8, 1989 which is incorporated herein by reference thereto teaches glycolipid analogues including N-glycosylamides, N-glycosylureas and N-glycosylcarbamates, each of which is substituted in the sugar residue by an amino acid, as immuno-modulators or adjuvants. Thus, Lockhoff et al. 1991 reported that N-glycolipid analogs displaying structural similarities to the naturally-occurring glycolipids, such as glycosphingolipids and glycoglycerolipids, are capable of eliciting strong immune responses in both herpes simplex virus vaccine and pseudorabies virus vaccine. Some glycolipids have been synthesized from long chain-alkylamines and fatty acids

that are linked directly with the sugars through the anomeric carbon atom, to mimic the functions of the naturally occurring lipid residues.

U.S. Patent No. 4,258,029 granted to Moloney, assigned to the assignee hereof and incorporated herein by reference thereto, teaches that octadecyl tyrosine hydrochloride (OTH) functions as an adjuvant when complexed with tetanus toxoid and formalin inactivated type I, II and III poliomyelitis virus vaccine. Also, Nixon-George et al. 1990, reported that octadecyl esters of aromatic amino acids complexed with a recombinant hepatitis B surface antigen, enhanced the host immune responses against hepatitis B virus.

Lipidation of synthetic peptides has also been used to increase their immunogenicity. Thus, Wiesmuller 1989, describes a peptide with a sequence homologous to a foot-and-mouth disease viral protein coupled to an adjuvant tripalmityl-s-glyceryl-cysteinylserylserine, being a synthetic analogue of the N-terminal part of the lipoprotein from Gram negative bacteria. Furthermore, Deres et al. 1989, reported *in vivo* priming of virus-lpecific cytotoxic T lymphocytes with synthetic lipopeptide vaccine which comprised of modified synthetic peptides derived from influenza virus nucleoprotein by linkage to a lipopeptide, N-palmityl-s-[2,3-bis(palmitylxy)-(2RS)-propyl-[R]-cysteine (TPC).

2. Immunoassays

The transferrin receptor, analogs and fragments thereof and/or peptides of the present invention are useful as immunogens, as antigens in immunoassays including enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known in the art for the detection of anti-bacterial, *Haemophilus*, TfR and/or peptide antibodies. In ELISA assays, the transferrin receptor, analogs, fragments and/or peptides corresponding to portions of

TfR protein are immobilized onto a selected surface, for example a surface capable of binding proteins or peptides such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed transferrin receptor, analogs, fragments and/or peptides, a nonspecific protein such as a solution of bovine serum albumin (BSA) or casein that is known to be antigenically neutral with regard to the test sample may be bound to the selected surface. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific bindings of antisera onto the surface. Preferably, the selected peptides are from the conserved regions of Table 2 or Table 3 to enhance the cross-species detection unless one particular bacterial species is to be detected. In that event, a polypeptide is selected which is unique to the TfR of that particular species. Normally, the peptides are in the range of 12 residues and up and preferably 14 to 30 residues. It is understood however, that a mixture of peptides may be used either as an immunogen in a vaccine or as a diagnostic agent. There may be circumstances where a mixture of peptides from the conserved regions and/or from the non-conserved regions are used to provide cross-species protection and/or diagnosis. In this instance, the mixture of peptide immunogens is commonly referred to as a "cocktail" preparation for use as a vaccine or diagnostic agent.

The immobilizing surface is then contacted with a sample such as clinical or biological materials to be tested in a manner conducive to immune complex (antigen/antibody) formation. This may include diluting the sample with diluents such as BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate for from 2 to 4 hours, at temperatures such as of the order

of 25° to 37°C. Following incubation, the sample-contacted surface is washed to remove non-immunocomplexed material. The washing procedure may include washing with a solution such as PBS/Tween, or a borate buffer.

Following formation of specific immunocomplexes between the test sample and the bound transferrin receptor, analogs, fragments and/or peptides, and subsequent washing, the occurrence, and even amount, of immunocomplex formation may be determined by subjecting the immunocomplex to a second antibody having specificity for the first antibody. If the test sample is of human origin, the second antibody is an antibody having specificity for human immunoglobulins and in general IgG. To provide detecting means, the second antibody may have an associated activity such as an enzymatic activity that will generate, for example, a color development upon incubating with an appropriate chromogenic substrate. Quantification may then achieved by measuring the degree of color generation using, for example, a visible spectra spectrophotometer.

3. Use of Sequences as Hybridization Probes

The nucleotide sequences of the present invention, comprising the sequence of the transferrin receptor gene, now allow for the identification and cloning of the transferrin receptor genes from any species of *Haemophilus* and other bacteria that have transferrin receptor genes.

The nucleotide sequences comprising the sequence of the transferrin receptor genes of the present invention are useful for their ability to selectively form duplex molecules with complementary stretches of other TfR genes. Depending on the application, a variety of hybridization conditions may be employed to achieve varying degrees of selectivity of the probe toward the other TfR genes. For a high degree of selectivity, relatively stringent conditions are used to form the

duplexes, such as low salt and/or high temperature conditions, such as provided by 0.02 M to 0.15 M NaCl at temperatures of between about 50°C to 70°C. For some applications, less stringent hybridization conditions are required such as 0.15 M to 0.9 M salt, at temperatures ranging from between about 20°C to 55°C. Hybridization conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilize the hybrid duplex. Thus, particular hybridization conditions can be readily manipulated, and will generally be a method of choice depending on the desired results. In general, convenient hybridization temperatures in the presence of 50% formamide are: 42°C for a probe which is 95 to 100% homologous to the target fragment, 37°C for 90 to 95% homology and 32°C for 85 to 90% homology.

In a clinical diagnostic embodiment, the nucleic acid sequences of the TfR genes of the present invention may be used in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of providing a detectable signal. In some diagnostic embodiments, an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of a radioactive tag may be used. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with samples containing TfR gene sequences.

The nucleic acid sequences of TfR genes of the present invention are useful as hybridization probes in solution hybridizations and in embodiments employing solid-phase procedures. In embodiments involving solid-phase procedures, the test DNA (or RNA) from samples,

such as clinical samples, including exudates, body fluids (e. g., serum, amniotic fluid, middle ear effusion, sputum, bronchoalveolar lavage fluid) or even tissues, is adsorbed or otherwise affixed to a selected matrix or surface. The fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes comprising the nucleic acid sequences of the TfR genes or fragments thereof of the present invention under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required depending on, for example, the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe etc. Following washing of the hybridization surface so as to remove non-specifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label. As with the selection of peptides, it is preferred to select nucleic acid sequence portions which are conserved among species of *Haemophilus*, such as nucleic acid sequences encoding the conserved peptide sequence of Figures 8, 9, 13 and 14 and particularly listed in Tables 2 and 3. The selected probe may be at least 18 bp and may be in the range of 30 bp to 90 bp long.

4. Expression of the Transferrin Receptor Genes

Plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell may be used for the expression of the transferrin receptor genes in expression systems. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* may be transformed using pBR322 which contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters

which can be used by the host cell for expression of its own proteins.

In addition, phage vectors containing replicon and control sequences that are compatible with the host can be used as a transforming vector in connection with these hosts. For example, the phage in lambda GEM™-11 may be utilized in making recombinant phage vectors which can be used to transform host cells, such as *E. coli* LE392.

Promoters commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems (Chang *et al.*, 1978: Itakura *et al.*, 1977 Goeddel *et al.*, 1979; Goeddel *et al.*, 1980) and other microbial promoters such as the T7 promoter system (U.S. Patent 4,952,496). Details concerning the nucleotide sequences of promoters are known, enabling a skilled worker to ligate them functionally with genes. The particular promoter used will generally be a matter of choice depending upon the desired results. Hosts that are appropriate for expression of the transferrin receptor genes, fragment analogs or variants thereof include *E. coli*, *Bacillus* species, *Haemophilus*, fungi, yeast or the baculovirus expression system may be used.

In accordance with this invention, it is preferred to make the protein by recombinant methods, particularly when the naturally occurring TfR protein as purified from a culture of a species of *Haemophilus* may include trace amounts of toxic materials or other contaminants . This problem can be avoided by using recombinantly produced TfR protein in heterologous systems which can be isolated from the host in a manner to minimize comtaminants in the purified material. Particularly desirable hosts for expression in this regard include Gram positive bacteria which do not have LPS and are therefore endotoxin free. Such hosts include species of *Bacillus* and may be particularly useful for the production of non-pyrogenic transferrin receptor, fragments or analogs thereof.

Furthermore, recombinant methods of production permit the manufacture of Tbp1 or Tbp2 or fragments thereof separate from one another which is distinct from the normal combined proteins present in *Haemophilus*.

Biological Deposits

Certain plasmids that contain at least a portion coding for a transferrin receptor from strains of *Haemophilus influenzae* that are described and referred to herein have been deposited with the American Type Culture Collection (ATCC) located at Rockville, Maryland USA pursuant to the Budapest Treaty and prior to the filing of this application. Samples of the deposited plasmids will become available to the public upon grant of a patent based upon this United States patent application. The invention described and claimed herein is not to be limited in scope by plasmids deposited, since the deposited embodiment is intended only as an illustration of the invention. Any equivalent or similar plasmids that encode similar or equivalent antigens as described in this application are within the scope of the invention.

Deposit Summary

Clone	ATCC Designation	Date Deposited
DS-712-1-3	75603	November 4, 1993
JB-1042-7-6	75607	November 4, 1993
JB-1424-2-8	75937	October 27, 1994
JB-1600-1	75935	October 27, 1994
JB-1468-29	75936	October 27, 1994
pT7TBP2A	75931	October 27, 1994
pT7TBP2B	75932	October 27, 1994
pT7TBP2C	75933	October 27, 1994
pT7TBP2D	75934	October 27, 1994

Strains of *Haemophilus*

Hib strain Eagan is available from Connaught Laboratories Limited, 1755 Steeles Ave. W., Willowdale, Ontario, Canada M2R 3T4.

Hib strain MinnA was obtained from the collection of Dr. Robert Munson, Department of Microbiology and Immunology, Washington University School of Medicine, Children's Hospital, St. Louis, Missouri 63110.

Hib strain DL63 was obtained from the collection of Dr. Eric Hansen, Department of Microbiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235-9048.

PAK 12085 was obtained from the collection of Dr. Robert Munson (supra).

SB12, 29, 30, 32 and 33 were obtained from the collection of Dr. Stephen Barenkamp, Department of Pediatrics, School of Medicine, Saint Louis University Medical Centre, St. Louis, Missouri 63104.

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics, protein biochemistry, immunology and fermentation technology used but not explicitly described in this disclosure and these Examples are amply reported in the scientific literature and are well within the ability of those skilled in the art.

Example 1

This Example illustrates the preparation of chromosomal DNA from *H. influenzae* strains DL63, Eagan, MinnA, and PAK 12085, and SB33.

H. influenzae strains were grown on Mueller-Hinton agar or in brain heart infusion broth as described by Harkness *et al* 1992.

A. Chromosomal DNA extraction from *Haemophilus influenzae* type b DL63

Chromosomal DNA was prepared as follows. Two hundred and fifty ml of culture were pelleted by centrifugation at 8,000 rpm in a Beckman J14 rotor for 15 minutes. The pellet was washed with 200 ml of 50mM Tris-HCl, pH 8.0, centrifuged as before, resuspended in 12.5 ml of 50mM Tris-HCl, 50mM EDTA, pH 8.0, and frozen at -20°C. Then 1.25 ml of a 10 mg/ml lysozyme solution in 0.25M Tris-HCl, pH 8.0, was added to the frozen cell pellet. The pellet was thawed and incubated on ice for 45 minutes. Next, 2.5 ml of a solution of 1mg/ml proteinase K in 0.5% SDS, 0.4M EDTA, 50mM Tris-HCl, pH 7.5 was added and the mixture incubated at 50°C for 1 hour with occasional mixing. The lysate was extracted once with 15 ml of Tris-buffered phenol, then 1.5 ml of 3M sodium acetate and 30 ml of ethanol were added to precipitate the DNA. The DNA was spooled on a glass rod, then dissolved in 12.5 ml of 50mM Tris-HCl, 1mM EDTA, pH 7.5 containing 0.2 mg/ml RNAse A by rocking overnight. The sample was extracted once with an equal volume of chloroform, precipitated, and spooled as above. The DNA was dissolved in 2 ml of 50mM Tris-HCl, 1mM EDTA, pH 7.5 and stored at 4°C.

B. Chromosomal DNA extraction from *Haemophilus influenzae* type b Eagan

Fifty ml of culture were pelleted by centrifugation, the pellet resuspended in 25ml of TE (10mM Tris, 1mM EDTA, pH 7.5), and 2 x 5ml aliquots used for chromosomal

DNA preparation. To each aliquot was added 0.6ml of 10% sarkosyl and 0.15ml of 20mg/ml proteinase K and the samples incubated at 37°C for 1 hour. The lysate was extracted once with Tris-saturated phenol and three times with chloroform:isoamyl alcohol (24:1). The aqueous phases were pooled for a final volume of 7ml. Then 0.7ml of 3M sodium acetate (pH 5.2) and 4.3 ml of isopropanol were added to precipitate the DNA which was spooled, rinsed with 70% ethanol, dried, and resuspended in 1 ml of water.

C. Chromosomal DNA extraction from *Haemophilus influenzae* Eagan, MinnA, PAK 12085 and SB33

Cells were pelleted from 50 ml of culture by centrifugation at 5000 rpm for 15-20 minutes, at 4°C. The cell pellet was resuspended in 10 ml of TE (10mM Tris-HCl, 1mM EDTA, pH 7.5), pronase and SDS were added to final concentrations of 500 μg/ml and 1%, respectively. The sample was incubated at 37°C for 4 hours until a clear lysate was obtained. The lysate was extracted once with Tris-saturated phenol, once with Tris-saturated phenol/chloroform (1:1), and once with chloroform. The final aqueous phase was dialysed for 24 hours against 2 x 500 ml of 1M NaCl at 4°C, changing the buffer once, and for 24 hours against 2 x 500 ml of TE at 4°C, changing the buffer once. The final dialysate was aliquotted for use.

Example 2

This Example illustrates the preparation of chromosomal libraries.

A. *H. influenzae* DL63-λZAP library

100 μg of *H. influenzae* DL63 chromosomal DNA in TE was mechanically sheared in a 1 ml syringe with a 25 gauge needle. The sheared DNA was made blunt-ended by adding water to a final volume of 405 μl, 45 μl of 10x S1 nuclease buffer (2M NaCl, 500mM NaOAc, pH 4.5, 10mM $ZnSO_4$, 5% glycerol), and 1.7 μl of S1 nuclease at 100

U/μl and incubating at 37°C for 15 min. The sample was extracted once with phenol/chloroform and once with chloroform and 1 ml of ethanol was added to precipitate the DNA. The sample was incubated on ice for 10 min or at -20°C overnight and the DNA was harvested by centrifugation in a microfuge for 30 minutes. The DNA was washed with 70% ethanol and dried. The *Eco* RI sites in the DNA sequence were methylated using standard procedures. To this methylated DNA was added 5 μl of 100mM $MgCl_2$, 8 μl of dNTP mix (2.5 mM each of dATP, dCTP, dGTP, and dTTP), and 4 μl of 5 U/μl Klenow. The mixture was incubated at 12°C for 30 minutes. 450 μl of STE (0.1M NaCl, 10mM Tris-HCl, 1mM EDTA, pH 8.0) were added, and the mixture extracted once with phenol/chloroform, and once with chloroform, before adding 1 ml of ethanol to precipitate the DNA. The sample was incubated on ice for 10 min or at -20°C overnight. The DNA was harvested by centrifugation in a microfuge for 30 minutes, washed with 70% ethanol and dried.

The DNA was resuspended in 7 μl of TE and to the solution was added 14 μl of phosphorylated *Eco* RI linkers (200 ng/μl), 3 μl of 10x ligation buffer, 3 μl of 10mM ATP, and 3μl of T4 DNA ligase (4 U/μl). The sample was incubated at 4°C overnight, then incubated at 68°C for 10 minutes to inactivate the ligase. To the mixture was added 218 μl of H_2O, 45 μl of 10x Universal buffer, and 7 μl of *Eco* RI at 30 U/μl. After incubation at 37°C for 1.5 hours, 1.5 μl of 0.5M EDTA was added, and the mixture placed on ice.

The DNA was size fractionated on a sucrose gradient, pooling fractions containing DNA of 6-10 kb. The pooled DNA was ethanol precipitated and resuspended in 5 μl of TE buffer. 200ng of insert DNA was ligated for 2-3 days at 4°C with 1 μg of ZAP II vector in a final volume of 5μl. The ligation mixture was packaged using Gigapack II Gold (Stratagene) and plated on *E. coli* SURE cells on NZY

plates. The library was titrated, amplified, and stored at 4°C under 0.3% chloroform.

B. *H. influenzae* Eagan-pUC library

Chromosomal DNA prepared from *H. influenzae* Eagan by the method in Example 1C was digested with *Sau*3A I for 2, 5, and 10 minutes and samples electrophoresed on a preparative agarose gel. Gel slices which included DNA fragments between 3-10 kb in length were excised and the DNA extracted by the standard freeze-thaw procedure. Plasmid DNA from pUC 8:2 (pUC 8 with additional *Bgl* II and *Xba* I restriction enzyme sites in the multiple cloning site) was digested with *BamH* I and *Bgl* II, and dephosphorylated with calf alkaline phosphatase (CAP). The fragments of *H. influenzae* Eagan DNA were ligated into pUC and the mixture used to transform *E. coli* JM109 cells.

C. *H. influenzae* Eagan-λZAP library

Chromosomal DNA from *H. influenzae* Eagan prepared as in Example 1B was digested with *Eco* RI and size fractionated on a preparative agarose gel. Gel slices corresponding to DNA fragments of 7-23 kb were excised and DNA was electroeluted overnight in dialysis tubing containing 3 ml of TAE (40mM Tris-acetate, 1mM EDTA) at 14V. The DNA was precipitated twice and resuspended in water before being ligated overnight with *Eco* RI digested λZAP II DNA. The ligation mixture was packaged using the Gigapack II packaging kit (Stratagene) and plated on *E. coli* XL1-Blue cells. The library was titrated, amplified, and stored at 4°C under 0.3% chloroform.

D. EMBL3 libraries

H. influenzae MinnA chromosomal DNA (10 μg) was prepared as in Example 1C and digested with *Sau*3A I (40 units) for 2, 4, and 6 minutes then size-fractionated on a 10-30% sucrose gradient in TNE buffer (20mM Tris-HCl, 5mM NaCl, 1mM EDTA, pH 8). Fractions containing DNA fragments greater than 5 kb were pooled and precipitated.

In a second experiment, chromosomal DNA (2.6 μg) was digested with *Sau*3A I (4 units) for 1, 2, and 3 minutes and size-fractionated by preparative agarose gel electrophoresis. Gel slices containing DNA fragments of 10-20 kb were excised and DNA extracted by a standard freeze/thaw technique. The size- fractionated DNA from the two experiments was pooled for ligation with *BamH* I arms of EMBL3 (Promega). The ligation mixture was packaged using the Gigapack II packaging kit and plated on *E. coli* LE392 cells. The library was titrated, then amplified and stored at 4°C under 0.3% chloroform.

Chromosomal DNA from *H. influenzae* PAK 12085 or SB33 prepared as in Example 1C was digested with *Sau*3A I (0.5 units/10 μg DNA) at 37°C for 15 minutes and size-fractionated by agarose gel electrophoresis. Gel slices corresponding to DNA fragments of 15-23 kb were excised and DNA was electroeluted overnight in dialysis tubing containing 3 ml of TAE at 14V. The DNA was precipitated twice and resuspended in water before overnight ligation with EMBL3 *BamH* I arms (Promega). The ligation mixture was packaged using the Lambda *in vitro* packaging kit (Amersham) according to the manufacturer's instructions and plated onto *E. coli* NM539 cells. The library was titrated, then amplified, and stored at 4°C in the presence of 0.3% chloroform.

Example 3

This Example illustrates screening of the libraries

A. *H. influenzae* DL63-λZAP expression library

Tbp1 and Tbp2 proteins were affinity purified on solid phase human transferrin (hTf). Briefly, a 20 ml hTf-Sepharose column was prepared according to the manufacturer's protocol for coupling protein ligands to CNBr-activated Sepharose (Sigma). The resulting matrix was washed with 3 column volumes of 50mM Tris-HCl, 1M NaCl, 6M guanidine-HCl, pH 8.0 to remove non-covalently bound hTf. The column was then equilibrated with 50mM

Tris-HCl, pH 8.0 and bound hTf was iron loaded using 1 ml of 10mg/ml $FeCl_3$ in buffer containing 100mM each of sodium citrate and sodium bicarbonate, pH 8.6, followed by 2 column volumes of 50mM Tris-HCl, 1M NaCl, pH 8.0. Total bacterial membranes (300 mg total protein) were prepared from *H. influenzae* strain DL63 grown on iron deficient media as described previously (Schryvers *et al.*, 1989). Membranes were diluted to 2 mg/ml in 50mM Tris-HCl, 1M NaCl, pH 8.0 and solubilized by the addition of EDTA to 15mM and Sarkosyl NL97 to 1.5%. After centrifugation at 40,000 x g for 1 hour, the supernatant was applied to the hTf column and the column washed with 10 column volumes of 50mM Tris-HCl, 1M NaCl, 10mM EDTA, 0.5% Sarkosyl, pH 8.0. The receptor proteins were eluted using 2M GnHCl in the same buffer and the eluted fractions were dialysed extensively against 25mM ammonium bicarbonate buffer (5 buffer changes), lyophilized, and stored at -20°C. Isolated proteins were used to generate transferrin receptor-specific antisera in New Zealand White rabbits using standard techniques. Briefly, rabbits were immunized 3 times subcutaneously, at intervals of two weeks, using complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for subsequent injections.

The DL63 λZAP library was plated on *E. coli* SURE cells and plaques were transferred onto nitrocellulose membranes which had been pre-soaked in 10mM IPTG to induce expression from the pBluescript lacZ promoter. Filters were blocked using 0.5% skim milk in 50mM Tris-HCl, 150mM NaCl, pH 7.5, prior to being probed with the polyclonal anti-TfR antisera and horse radish peroxidase-conjugated goat anti-rabbit IgG. Plaques were purified by 3 rounds of screening and recombinant pBluescript plasmids (pBHIT1 and pBHIT2; Figures 1A and 2) were recovered by the *in vivo* excision procedure (Short *et al.*, 1988).

B. Eagan, MinnA, and PAK 12085 non-expression libraries

(i) Screening of *H. influenzae* Eagan-pUC library

Colony lifts onto nitrocellulose were performed using standard techniques and the filters were probed with the 5'pBHIT2 probe of the transferrin receptor gene illustrated in Figure 2. The probe was labelled with digoxigenin (dig, Boehringer Mannheim) following the manufacturer's specifications. Several putative clones were dot blotted onto nitrocellulose and submitted to second round screening using the same 5'pBHIT2 probe. Second round putatives were analysed by restriction enzyme mapping and clone S-4368-3-3 (Figure 1B, Figure 2) was selected for sequence analysis.

(ii) Screening *H. influenzae* Eagan-λZAP library

The phage library was plated using standard techniques on XLI Blue cells (Stratagene) using LB plates and a 0.7% agarose overlay layer. Plaques were lifted onto nitrocellulose using standard protocols and the filters were baked at 80°C, for 2 hours, under vacuum, to fix the DNA. The 5'pBHIT2 probe of the transferrin receptor gene (Figure 2) was labelled with digoxigenin and the filters were pre-hybridized for 4 hours at 42°C, then hybridized with the labelled probe at 42°C, overnight. The filters were washed at 68°C and after autoradiography, several plaques were selected for second round screening. *In vivo* excision of phagemid DNA from second round putatives was performed according to protocols provided with the λZAP system (Promega). Four clones with identical ~2.5 kb *Eco* RI inserts were obtained of which JB-901-5-3 in Figure B, Figure 2 is an example. Putative plaques were also amplified and phage DNA was purified from 500 ml of culture. Insert DNA was excised by digestion with *Xba* I and was cloned into pUC 8:2 (pUC 8 containing additional *Bgl* II and *Xba* I sites in its multiple cloning site) which had been digested with *Xba* I and dephosphorylated. Clone JB-911-3-2

(Figure 17) contains the 3'-half of the *H. influenzae* Eagan TfR operon.

(iii) Screening EMBL 3 libraries

The *H. influenzae* MinnA library was plated onto LE392 cells on NZCYM plates using 0.7% top agarose in NZCYM as overlay. Plaque lifts onto nitrocellulose filters were performed following standard procedures, and filters were processed and probed with the 5'pBHIT2 probe (Figure 2) labelled with digoxigenin. Putative plaques were plated and submitted to second and third rounds of screening using the same procedures. Phage DNA was prepared from 500 ml of culture using standard techniques, the insert DNA excised by *Sal* I digestion, and cloned into pUC to generate clone DS-712-1-3 (Figures 1C and 2).

The *H. influenzae* PAK 12085 library was plated on LE392 cells on NZCYM plates using 0.7% agarose in NZCYM as overlay. Plaques were lifted onto nitrocellulose and filters were processed and probed with the digoxigenin-labelled 5'pBHIT2 probe (Figure 2). Putative plaques were plated and subjected to a second round of screening using the same procedures. Phage DNA was prepared from 500 ml cultures by standard techniques, the DNA insert was excised by digestion with *Sal* I, and cloned into pUC to generate clone JB-1042-7-6 (Figure 1D and 2).

The *H. influenzae* SB33 library was plated on LE392 cells on NZCYM plates using 0.7% agarose in NZCYM as overlay. Plaques were lifted onto nitrocellulose and filters were processed and probed with the digoxigenin-labelled 5'pBHIT2 probe (Figure 2). Putative plaques were plated and subjected to a second round of screening using the same procedures. Phage DNA was prepared from 500 ml cultures by standard techniques, the DNA insert was excised by digestion with *Sal* I, and cloned into pUC to generate clone JB-1031-2-9 (Figure 2).

Example 4

This Example illustrates the sequencing of the Tbp1 and Tbp2 genes of the TfR operon.

Plasmid DNA from clones pBHIT 1, pBHIT 2, S-4368-3-3, JB-901-5-3, DS-712-1-3, JB-1042-7-6 and JB-1031-2-9 was prepared using standard techniques. Oligonucleotide sequencing primers of 17-25 bases in length were synthesized on the ABI model 380B DNA Synthesizer and purified by chromatography using OPC cartridges obtained from Applied Biosystems Inc., and used in accordance with the manufactures recommendations. Samples were sequenced using the ABI model 370A DNA Sequencer and dye terminator chemistry according to manufacturers' protocols. The sequence of the TfR operon from strain DL63 is illustrated in Figure 3, that of strain Eagan in Figure 4, that of strain MinnA in Figure 5, that of PAK 12085 in Figure 6 and that of SB33 in Figure 7.

Example 5

This Example illustrates the PCR amplification of the *tbp2* genes from non-typable *H. influenzae* strains SB12, SB29, SB30, and SB32.

Chromosomal DNA from non-typable *H. influenzae* strains SB12, SB29, SB30, and SB32 was prepared as described aobve. The TfR genes are arranged as an operon with *tbp2* followed by *tbp1* (see Figures 12A and 12B). Oligonucleotides were synthesized to the 5'-end of the *tbp2* and the reverse complement of the 5'-end of the *tbp1* coding sequences. The primers were: GGATCCATATGAAATCTGT ACCTCTTATCTCTGGT (SEQ ID NO: 120) corresponding to MKSVPLISGS (SEQ ID NO: 147) from the leader sequence of Tbp2 and TCTAGAAGCTTTTTTAGTCATTTTTAGTATTCCAT (SEQ ID NO: 137) which is the reverse complement of the leader sequence MTKK (SEQ ID NO: 138) of Tbp1 and a part of the intergenic sequence (Figures 12A and 12B). PCR amplification was performed in buffer containing 10mM Tris/HC1 pH 8.3, 50 mM potassium chloride and 1.5 mM magnesium chloride. Each 100 μl reaction mixture

contained 5 ng of chromosomal DNA, 1 μg of each primer, 5 units amplitaq DNA polymerase (Perkin Elmer Cetus) and 0.4 mM dNTPs (Perkin Elmer Cetus). The cycling conditions were 25 cycles of 94°C for 1.0 min, 45°C for 2.0 min and 72°C for 1.5 min. Specific 2 kb fragments were amplified for each sample (Figure 13). SB33 DNA was used as a positive control (Lane 1). Chromosomal DNA used for amplification of the Tbp2 gene were lane 1, SB33; lane 2, SB12; lane 3, SB29; lane 4, SB30; and lane 5, SB32. The fragments were cloned into the TA cloning vector (Invitrogen) and their nucleotide sequences determined. The nucleic acid sequences of Tbp2 from strains SB12 (SEQ ID NO: 108), SB29 (SEQ ID NO: 110), SB30 (SEQ ID NO: 112) and SB32 (SEQ ID NO: 114) are shown in Figures 8, 9 10 and 11 respectively.

Example 6

This Example illustrates the comparison of the amino acid sequences of transferrin the identification of potentially exposed epitopes of transferrin receptor proteins by secondary structure analysis.

Referring to Figure 14, there is shown a comparison of the amino acid sequence of Tbp1 from *H. influenzae* type b Eagan, DL63, non-typable *H. influenzae* strains PAK 12085 and SB33, *N. meningitidis* strains B16B6 and M982 (Legrain *et al.*, 1993) and *N. gonorrhoeae* FA19 (Cornelissen *et al.*, 1992). This analysis revealed regions of Tbp1 which are conserved among all these bacteria.

Referring to Figure 15, there is shown a comparison of the amino acid sequence of Tbp2 from *H. influenzae* type b strains Eagan, DL63, non-typable *H. influenzae* PAK 12085, SB12, SB29, SB30 and SB32, *N. meningitidis* strains B16B6 and M982, *N. gonorrhoeae* FA19 and *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* (Gerlach *et al.*, 1992) 205 and 37. This analysis revealed regions of Tbp2 which are conserved among all these bacteria.

Protein secondary structure analyses were performed using the Chou and Fasman algorithms (1978) and hydrophilicity/hydrophobicity plots were performed using the Hopp algorithm (1986). The values were derived from the averages of heptapeptide windows and are plotted at the midpoint of each fragment. Figure 16A illustrates the predicted secondary structure of Tbp1 from *H. influenzae* type b Eagan and Figure 16B illustrates the predicted secondary structure of Tbp2 from *H. influenzae* type b Eagan. The predicted secondary structures depicted in Figures 16A and 16B were arrived at using the procedures described above. However, the inventors have not yet been able to verify that the secondary structure is accurately depicted by these Figures.

Conserved epitopes of Tbp1 and Tbp2 proteins from several different bacteria were identified by sequence alignment as shown in Figures 14 and 15 respectively. Some such conserved epitopes include:

TBP1	DNEVTGLGK	SEQ ID NO:43
TBP1	EQVLNIRLTRYDPGI	SEQ ID NO:44
TBP1	GAINEIEYENVKAVEISKG	SEQ ID NO:45
TBP1	GALAGSV	SEQ ID NO:46
TBP2	LEGGFYGP	SEQ ID NO:74
TBP2	CSGGGSFD	SEQ ID NO:75
TBP2	YVYSGL	SEQ ID NO:76
TBP2	CCSNLSYVKFG	SEQ ID NO:77
TBP2	FLLGHRT	SEQ ID NO:78
TBP2	EFNVDF	SEQ ID NO:79
TBP2	NAFTGTA	SEQ ID NO:80
TBP2	VNGAFYG	SEQ ID NO:81
TBP2	LEGGYF	SEQ ID NO:82
TBP2	VVFGAR	SEQ ID NO:83

Furthermore, in combination with the predicted secondary structures, four conserved exposed epitopes were identified on Tbp1 and two were identified on Tbp2. These are:

Tbp1	DNEVTGLGK	SEQ ID NO:43
Tbp1	EQVLN/DIRDLTRYD	SEQ ID NOS: 139 and 140
Tbp1	GAINEIEYENVKAVEISK	SEQ ID NO:141
Tbp1	GI/VYNLF/LNYRYVTWE	SEQ ID NOS:142 and 143
Tbp2	CS/LGGG(G)SFD	SEQ ID NOS: 75, 144 and 145
Tbp2	LE/SGGFY/FGP	SEQ ID NOS: 74 and 146

Proteins, polypeptides or peptides containing the afore-mentioned conserved amino acid sequences are particularly useful as detecting means in diagnostic embodiments and as immunogens to detect or protect from diseases caused by bacteria that produce transferrin receptor protein. For immunization, the particularly indicated amino acid sequences may be presented to the immune system as proteins or peptides or a live delivery vehicle, such as *Salmonella*, BCG, adenovirus, poxvirus, vaccinia or poliovirus may be used.

Example 7

This Example illustrates the construction of plasmid JB-1468-29 which expresses Eagan Tbp1 from *E.coli*.

Plasmids S-4368-3-3 (Figures 1B and 2) and JB-911-3-2 (Figure 17) contain the 5'- and 3'- parts of the Eagan *tbp1* gene, respectively. Figure 17 illustrates the construction scheme for plasmid JB-1468-29. The oligonucleotide sequences used in the construction of JB-1468-29 are shown in Figure 20, (SEQ ID NOS: 86 and 87). Plasmid JB-1468-29 was introduced into *E. coli* strain BL21/DE3 by electroporation to generate strain JB-1476-2-1.

JB-1476-2-1 was grown in YT medium and induced with IPTG following standard protocols. For preparation of Tbp1 for immunogenicity and other studies, strain JB-1476-2-1 was grown overnight in NZCYM media containing 3% glucose. A 1:40 inoculum was added to fresh NZCYM media without glucose, and the culture grown to A_{578}=0.3. Lactose was added to 1% and the culture was induced for 4 hours. SDS-PAGE analysis of whole cell lysates of JB-

1476-2-1 is shown in Figure 22. Lane 1, JB-1476-2-1 (T7/Eagan Tbp1) at t_o; lane 2, JB-1476-2-1 at t=4h induction; lane 3, molecular weight markers of 200 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa and 31 kDa; lane 4, JB-1437-4-1 (T7/Eagan Tbp2) at t_o; lane 5, JB-1437-4-1 at t=4h induction; lane 6, JB-1607-1-1 (T7/SB12 Tbp2) at t_o; lane 7, JB-1607-1-1 at t=4h induction.

Example 8

This Example illustrates the construction of plasmid JB-1424-2-8 which expresses Eagan Tbp2 from *E. coli*.

Referring to Figure 18, there is shown plasmid S-4368-3-3 which contains the entire *tbp2* gene from *H. influenzae* type b Eagan. Figure 18 illustrates plasmid JB-1424-2-8 and Figure 19 shows the oligonucleotides used. Plasmid JB-1424-2-8 was introduced into *E. coli* strain BL21/DE3 by electroporation to generate *E. coli* strain JB-1437-4-1. Upon induction with IPTG or lactose, Tbp2 was expressed by *E. coli* JB-1437-4-1 as shown in Figure 22. Lane 1, JB-1476-2-1 (T7/Eagan Tbp1) at t_o; lane 2, JB-1476-2-1 at t=4h induction; lane 3, molecular weight markers of 200 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa and 31 kDa; lane 4, JB-1437-4-1 (T7/Eagan Tbp2) at t_o; lane 5, JB-1437-4-1 at t=4h induction; lane 6, JB-1607-1-1 (T7/SB12 Tbp2) at t_o; lane 7, JB-1607-1-1 at t=4h induction.

Example 9

This Example illustrates the construction of plasmids which encode a lipoprotein leader sequence before the Tbp2 sequence.

Oligonucleotides used for the construction of plasmids with lipoprotein leader sequences derived from *E. coli* lpp (SEQ ID NOS: 88 and 89), rlpB (SEQ ID NOS: 90 and 91), and pal (SEQ ID NOS: 92 and 93) preceeding Tbp2 are shown in Figure 20. Plasmids constructed and corresponding strains generated are illustrated in Table 1 below.

Example 10

This Example illustrates the construction of plasmid JB-1600-1 which expresses SB12 Tbp2 from *E. coli*.

Plasmid DS-1047-1-2 (Figure 21) contains the PCR-amplified SB12 *tbp2* gene. The *tbp2* gene was excised as a Nde I to EcoR I restriction fragment and inserted into the pT7-7 expression vector to generate plasmid JB-1600-1. Electroporation into BL21/DE3 cells yielded *E. coli* strain JB-1607-1-1 which expresses SB12 Tbp2. Upon induction with IPTG or lactose, SB12 Tbp2 was expressed, as shown in Figure 22. Lane 1, JB-1476-2-1 (T7/Eagan Tbp1) at t_o; lane 2, JB-1476-2-1 at t=4h induction; lane 3, molecular weight markers of 200 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa and 31 kDa; lane 4, JB-1437-4-1 (T7/Eagan Tbp2) at t_o; lane 5, JB-1437-4-1 at t=4h induction; lane 6, JB-1607-1-1 (T7/SB12 Tbp2) at t_o; lane 7, JB-1607-1-1 at t=4h induction.

Example 11

This Example illustrates the extraction and purification of Tbp1 and Tbp2.

The purification scheme for Tbp1 and Tbp2 is shown in Figure 23. Both recombinant proteins are expressed as inclusion bodies in *E.coli* and the purification schemes are identical. Cells from a 500 ml culture, prepared as described in Example 7 for Tbp1 and in Example 8 for Tbp2, were resuspended in 50 ml of 50mM Tris-HCl, pH 8.0, and disrupted by sonication (3 x 10 min, 70% duty circle). The extract was centrifuged at 20,000 x *g* for 30 min and the resultant supernatant which contained > 95% of the soluble *E. coli* proteins was discarded.

The remaining pellet (Figure 23, PPT_1) was further extracted in 50 ml of 50 mM Tris, pH 8.0 containing 0.5% Triton X-100 and 10 mM EDTA. After centrifugation at 20,000 x *g* for 30 min, the supernatant containing residual soluble proteins and the majority of the membrane proteins, was discarded. The resultant pellet

(Figure 23, PPT_2) obtained after the above extraction, contained the inclusion bodies. The Tbp1 and Tbp2 proteins were solubilized in 50 mM Tris, pH 8.0, containing 0.1% SDS and 5 mM DTT. After centrifugation, the resultant supernatant was further purified on a Superdex 200 gel filtration column equilibrated in 50 mM Tris, pH 8.0, containing 0.1% SDS and 5mM DTT. The fractions were analysed by SDS PAGE and those containing purified Tbp1 or Tbp2 were dialysed overnight at 4°C against 50 mM Tris, pH 8.0 and then centrifuged at 20,000 x *g* for 30 min. The protein remained soluble under these conditions and the purified Tbp1 and Tbp2 were stored at -20°C.

The SDS-PAGE analysis of the purification process is shown in Figure 24. Lanes 1, prestained molecular weight protein markers (106, 80, 49.5, 32.5, 27.5, 18.5 kDa); lanes 2, *E.coli* whole cell lysates; lanes 3, solubilized inclusion bodies; lanes 4, purified Tbp1 or Tbp2.

In an alternative method for extraction and purification of recombinant rTbp1 and rTbp2, cells from a 500 mL culture, prepared as described in Example 7 for Tbp1 and Example 8 for Tbp2, were resuspended in 40 mL of 50 mM Tris-HCl, pH 8.0, containing 5 mM AEBSF (4-(2-aminoethyl)benzenesulfonylfluoride) and disrupted by sonication (3x 10 min, 70% duty circle). After centrifugation at 20,000 x g for 30 min, the resultant pellet was further extracted at room temperature for 1 hour in 40 mL 50 mM Tris-HCl, pH 8.0, containing 10 mM EDTA and 0.5% Triton X-100. After centrifugation as described above, the resultant pellet contained the rTbp1 or rTbp2 inclusion bodies. rTbp1 or rTbp2 was then solubilized in 50 mM Tris-HCl, pH 8.0, containing 6 M guanidine and 5 mM DTT. After centrifugation, the resultant supernatant was further purified on a Superdex 200 gel filtration column equilibrated in 50 mM Tris-HCl, pH 8.0, containing 2 M guanidine and 5 mM DTT. The

fractions were analyzed by SDS-PAGE and those containing purified rTbp1 or rTbp2 were pooled. Triton X-100 was added into the pooled solution to a final concentration of 0.1% and the solution was dialyzed against 50 mM Tris-HCl, pH 8.0 overnight at 4°C. The protein remained soluble under these conditions and the purified rTbp1 and rTbp2 were stored at 4°C.

Example 12

This Example illustrates immunogenicity studies of recombinant Tbp1 and Tbp2 in mice.

Groups of five Balb/c mice were injected subcutaneously (s.c.) on days 1, 29 and 43 with purified rTbp1 or rTbp2 (1 μg to 10 μg), prepared as described in Example 11, in the presence of $AlPO_4$ (1.5 mg per dose). Blood samples were taken on days 14, 28, 42 and 56 for analysis of the anti-rTbp1 and anti-rTbp2 antibody titers by EIA. The results of the immunogenicity studies are illustrated in Figure 25.

Example 13

This Example illustrates the development of EIAs for determination of anti-rTbp1 and anti-rTbp2 antibodies in mouse sera.

Anti-rTbp1 and anti-rTbp2 antibody titres were determined essentially as described by Panezutti *et al.* (1993). Microtiter wells were coated with 0.5 μg of rTbp1 or rTbp2, prepared as described in Example 11, for 16 h at room temperature, then blocked with 0.1% (w/v) BSA in PBS. The sera were serially diluted, added to the wells, then incubated for one hour at room temperature. Affinity-purified $F(ab')_2$ fragments of goat anti-mouse IgG (Fc specific) antibody conjugated to horseradish peroxidase were used as second antibody. The reactions were developed using tetramethylbenzidine (TMB/H_2O_2) and the absorbance was measured at 450 nm (using 540 nm as a reference wavelength) in a Flow Multiskan MCC microplate reader. The reactive titer of an antiserum was defined

as the reciprocal of the dilution consistently showing a two-fold increase in absorbance over that obtained with the pre-immune serum sample.

Example 14

This Example illustrates the cross-reactivity of anti-Tbp1 antisera, produced by immunization with recombinant Eagan Tbp1, with various strains of *H. influenzae*.

Whole cell lysates of *H. influenzae* strains grown in BHI media supplemented with NAD and heme (Harkness et al., 1992) ± EDDA were separated by SDS PAGE gel, transferred to nitrocellulose membrane, and probed with guinea pig anti-Tbp1 antisera raised to purified recombinant Eagan Tbp1 (Figure 26). Lane 1, BL21/DE3; lane 2, SB12-EDDA; lane 3, SB12 +EDDA; lane 4, SB29 -EDDA; lane 5, SB29 +EDDA; lane 6, SB33 -EDDA; lane 7, SB33 + EDDA; lane 8, Eagan -EDDA; lane 9, Eagan +EDDA; lane 10, *B. catarrhalis* 4223 - EDDA; lane 11, *B. catarrhalis* 4223 +EDDA; lane 12, *N. meningitidis* 608 - EDDA; lane 13, *N. meningitidis* 608 + EDDA; lane 14, induced JB-1476-2-1 expressing recombinant Eagan Tbp1; lane 5, molecular weight markers. Specific ~ 95 kDa bands reacted with the anti-Tbp1 antisera in lanes 3, 4, 5, 7, 8 and 9, corresponding *H. influenzae* strains SB12, SB29, SB33 and Eagan; ~ 110 kDa bands in lanes 10 and 11, corresponding *B. catarrhalis* strain 4223; and ~ 80 kDa bands in lanes 12 and 13, corresponding to *N. meningitidis* 608.

Example 15

This Example illustrates the cross-reactivity of anti-Tbp2 antisera, produced by immunization with recombinant Eagan Tbp2, with various strains of *H. influenzae*.

Whole cell lysates of *H. influenzae* strains grown in BHI media supplemented with NAD and heme (Harkness et al., 1992) ± EDDA were separated on an SDS PAGE gel,

transferred to nitrocellulose membrane, and probed with guinea pig anti-Tbp2 antisera raised to purified recombinant Eagan Tbp2 (Figure 27). Lane 1, molecular weight markers; lane 2, induced JB-1437-4-1 expressing recombinant Eagan Tbp2; lane 3, SB12-EDDA; lane 4, SB12 +EDDA; lane 5, SB29 -EDDA; lane 6, SB29 +EDDA; lane 7, SB30 -EDDA; lane 8, SB30 +EDDA; lane 9, SB32 -EDDA; lane 10, SB33-EDDA; lane 11, SB33 +EDDA; lane 12, PAK -EDDA; lane 13, PAK +EDDA; lane 14, Eagan -EDDA; lane 15, Eagan +EDDA. Specific bands of about 60-70 kDa were reactive with the anti-Tbp2 antisera in lanes 3, 6, 7, 8, 13, 14 and 15, corresponding to *Haemophilus* strains SB12, SB29, SB30, PAK and Eagan.

Example 16

This Example illustrates the synthesis of synthetic peptides corresponding to conserved regions in Tbp2 and Tbp1.

The deduced amino acid sequences of Tbp1 and Tbp2 were compared as shown in Figures 14 and 15 respectively. This comparison identified regions of amino acid sequence conservation within the transferrin receptor described above and, as shown in Tables 2 and 3, peptides were synthesized containing a portion of the transferrin receptor. Such synthesis may be effected by expression in a suitable host of recombinant vectors containing nucleic acid encoding said peptides or by standard peptide synthesis.

Briefly, peptides were synthesized using an ABI 430A peptide synthesizer and optimized t-Boc chemistry using the conditions recommended by the manufacturer, and peptides were cleaved from the resin using hydrofluoric acid (HF). The peptides were purified by reverse-phase high performance liquid chromatography (RP-HPLC) on a Vydac C4 semi-preparative column (1 x 30 cm) using a 15 to 55% acetonitrile gradient in 0.1% trifluoryl acetic acid (TFA) developed over 40 minutes at a flow rate of

2ml/minute. All synthetic peptides used in biochemical and immunological studies were >95% pure as judged by analytical HPLC. Amino acid composition analyses were performed on a Waters Pico-Tag system and were in good agreement with the theoretical compositions.

Example 17

This Example illustrates the immunogenicity of synthetic peptides in test animals.

Guinea pigs were immunized intramuscularly with 100 μg of peptide, prepared as described in Example 16, emulsified in Freund's complete adjuvant on day 0 followed by boosters on days + 14 and + 28 using the same amount of peptide emulsified in Freund's incomplete adjuvant. Sera samples were obtained on day 42 + and antibody titres determined by enzyme-linked immunosorbent assay (ELISA). Briefly, microtiter wells (Nunc-Immunoplate, Nunc, Denmark) were coated with 500 ng of any one particular peptide in 50 μL of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) for 16 hours at room temperature. The plates were then blocked with 0.1% (w/v) BSA in phosphate buffer saline (PBS) for 30 minutes at room temperature. The antisera were serially diluted, added to the wells and incubated for 1 hour at room temperature. After removal of the antisera, the plates were washed five times with PBS containing 0.1% (w/v) Tween-20 and 0.1% (w/v) BSA. $F(ab')_2$ from goat anti-guinea pig IgG antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Labs Inc., PA) were diluted (1/8,000) with washing buffer, and added onto the microtiter plates. After 1 hour of incubation at room temperature, the plates were washed five times with the washing buffer. The plates were developed using the substrate tetramethylbenzidine (TMB) in H_2O_2 (ADI, Toronto), the reaction was stopped with 1N H_2SO_4 and the optical density was measured at 450 nm using a Titretek Multiskan II (Flow Labs., Virginia). Two irrelevant

peptides of 32 amino acid residues were included as negative controls in these ELISAs. Assays were performed in triplicate, and the reactive titer of each antiserum was defined as the dilution consistently showing a 2-fold increase in absorbance value over those obtained from the negative controls. The antisera raised in guinea pigs were monospecific for the peptide used for immunization. The titres of the sera obtained following immunization are shown in Table 4.

Peptides of the present invention comprise single copies of any of those shown in Tables 2 and 3 or peptides containing multiple copies of analogs thereof. A peptide may further comprise multiples of different peptides selected from those shown in Tables 2 and 3 or analogs thereof and include suitable carrier molecules. It is preferred that the peptides from conserved regions be used to develop antibodies because an immuno- or other type of binding assay can then be used to detect several species of *Haemophilus*. Tables 2 and 3 therefore set out several other conserved regions of transferrin receptor to identify other peptides which would be useful in diagnosis, immunization and medical treatment.

Guinea pig anti-Eagan rTbp1, anti-Eagan rTbp2, and anti-SB12 rTbp2 antisera were used to screen a panel of *H. influenzae* strains for antigenic conservation of the Tbp1 and Tbp2 proteins. Of 33 strains screened by Western blot with anti-Eagan rTbp1 antisera, all had a reactive band of ~100 kDa. Of 89 strains screened by Western blot with anti-Eagan rTbp2 antisera, 85 had a reactive band of 60-90 kDa. Of 86 strains screened by Western blot with anti-SB12 rTbp2 antisera, 82 had a reactive band of 60-90 kDa. Only one strain was not recognized by either anti-Eagan rTbp2 or anti-SB12 rTbp2 antisera, and that was NTHi strain SB33 which has a defective *tbp*B gene. These data indicate that transferrin receptor proteins are highly conserved in strains of *H. influenzae* and support the

use of these proteins as antigens and in immunogenic compositions, including vaccines, for immunization against disease cause by *H. influenzae* and diagnosis thereof.

Example 18

This Example describes the ability of antiserum raised against peptides corresponding to conserved portions of transferrin receptor to recognize the transferrin receptor of *Branhamella catarrhalis*.

Guinea pigs were immunized with peptide, corresponding to conserved portions of transferrin receptor, and antisera obtained are described in Example 17. A whole-cell extract of *Branhamella catarrhalis* was immunoblotted with the peptide-specific antiserum which specifically recognized the transferrin receptor from this bacterium. Anti-peptide antiserum from a guinea pig immunized with the Tbp2 N-terminal peptide and peptide TBP2-25 specifically recognized Tbp2 protein from *Branhamella catarrhalis* and recombinant Tbp2 expressed by plasmid clone pBHIT2 in *E. coli*. Clone pBHIT2 expresses a truncated version of Tbp2 starting at amino acid 80. (i.e. NKKFYSG SEQ ID NO: 105). Therefore, the Tbp2 protein from pBHIT2 can only be recognized by antibodies raised against the second epitope LEGGFYGP (TBP2-25). This analysis shows that peptides corresponding to conserved sequences between transferrin receptor are useful in detecting most, if not all, bacteria that produce transferrin receptor and as components in immunogenic compositions, including vaccines to produce an immune response against transferrin receptor and protect against diseases caused by such bacteria.

The sera from these rabbits were tested by ELISA against a peptide incorporating the sequence LEGGFYGP (SEQ ID NO:74) or against *H. influenzae* strain DL63, Tbp2. ELISA plates were coated with the peptide or the protein then blocked with 5% skim milk. Serial two-fold dilutions of sera in phosphate buffered saline, 0.05%

tween-20, and 1% dried milk were incubated on the plates for two hours at 37°C, following which the plates were washed five times in phosphate buffered saline with 0.05% tween-20. Washed plates were probed with a horse-radish peroxidase (HRPO)-conjugated donkey anti-rabbit IgG for 30 minutes at room temperature, then washed five times in phosphate buffered saline with 0.05% tween-20. HRPO-substrate was added to all wells for 30 minutes at room temperature in the dark, then color developemnt was halted by the addition of 50 ul 1M sulphuric acid. Color was measured by determining absorbance at 450nm.

Example 19

This Example illustrates the generation of *H. influenzae* strains that do not produce transferrin receptor.

A 2.55 *Eco* RI fragment of the insert from pBHIT1 was subcloned into the *Eco* RI site of pUC4K, resulting in removal of the Tn903 kanamycin resistance (kan) cassette from this vector (pUHIT1; Figure 28). This subcloning step facilitated the subsequent insertion of either a HincII or PstI pUC4K fragment containing the kan cassette into the *Hind* III or *Pst* I sites of pUHIT1 as both are unique sites in this construction to produce pUHIT1KFH and pUHIT1KFP, Figure 28. Following digestion with *Eco* RI to remove the interrupted gene sequences, the constructs were introduced into the *H. influenzae* wild type genome by transformation using M-IV media as described previously (Barcak *et al.*, 1991) and transformants were selected on BHINH agar containing 20 μg/ml kanamycin.

Example 20

This Example illustrates the construction of polioviruses expressing an epitope of a transferrin receptor.

A cDNA clone of bases 1175 to 2956 of the poliovirus type 1, Mahoney strain (PV1-M) genome was cut with

restriction enzymes *Sau* I and *Hind* III. These enzymes excise a fragment containing bases 2754 to 2786, which encodes PV1-M amino acids 1094 to 1102, as shown in Figure 29. In this application, we use the four-digit code for poliovirus amino-acids; for example, 1095 is amino acid 95 of capsid protein VP1. New hybrid cDNA clones encoding both poliovirus and transferrin receptor amino-acid sequences were constructed by replacing the excised fragment with synthetic oligonucleotides coding for amino acids from *H. influenzae* Tbp2. The new hybrid cDNA clones were cut with restriction enzymes Nhe I and SnaB I, which excise a hybrid fragment, including the transferrin receptor DNA sequences, from poliovirus base 2471 to 2956. A cDNA clone, for example pT7XLD or pT7CMCB, of the entire genome of PV1-M was cut with Nhe I and SnaBI to excise a fragment from poliovirus base 2471 to 2956. This was then replaced with a hybrid fragment including the transferrin receptor DNA sequences to produce a hybrid cDNA clone of the genome of PV1-M with bases 2754 to 2786 replaced by bases encoding a hybrid BC loop including transferrin receptor amino acids, as shown in Figure 29.

The plasmid pT7XLD and clones derived from pT7XLD, such as pT7CMCB, contain a promoter sequence for the enzyme T7 RNA polymerase at the 5' end of the PV1-M cDNA. RNA transcripts of the PV1-M cDNA, including any bases encoding transferrin receptor amino acids, were prepared using T7 RNA polymerase as described by van der Werf et al. Transfection of Vero cells with these RNA transcripts produced four viable hybrid viruses, designated PV1TBP2A, PV1TBP2B, PV1TBP2C and PV1TBP2D. Transfection with transcripts of pT7CMCB yielded a transfection-derived wild-type poliovirus designated PV1XLD (Figure 29).

The antigenic characteristics of PV1TBP2A, PV1TBP2B, PV1TBP2C and PV1TBP2D are shown in Table 5. All were

neutralized by guinea-pig antisera raised against a peptide incorporating the sequence LEGGFYGP (SEQ ID NO: 74), indicating that the viruses expressed this sequence in an antigenically recognisable form. To produce the antisera female guinea pigs were immunized IM with a 500ul volume containing 200 ug peptide formulated in aluminum phosphate (3mg/ml). Animals were immunized on days 1, 14, 28 and 42 and bled on days 0, 28, 42 and 56. Sera were from the day 56 bleed. PV1TBP2A and PV1TBP2B were also neutralized by rabbit antisera raised against *H. influenzae* strain DL63 Tbp2, indicating that at least these two viruses expressed the sequence in a form recognisable to antibodies raised against the protein. All viruses were neutralisable by anti-PV1 sera, indicating that the changes in polio neutralization antigenic site I had not significantly affected other antigenic sites on the viruses.

Example 21

This Example illustrates the use of poliovirus hybrids to induce high titer antisera against Tbp2.

Rabbits were inoculated with CsCl-purified PV1TBP2A (rabbits #40, 41, 42). Note that, although the viruses used were live, poliovirus does not replicate in rabbits and that any response observed is effectively the response to an inactivated antigen. On day 1, rabbits were inoculated with 1 ug of virus in Freund's complete adjuvant subcutaneously on the back, and, on day 14, the rabbits were boosted with 1 ug of virus in Freund's incomplete adjuvant inoculated subcutaneously on the back. The rabbits were bled on day 0 (prebleed) and on day 27. The dose of virus per inoculation was 2.5×10^8 pfu, which was determined from A_{260} values to be approximately 3.0×10^{11} virions. This equivalent to 0.5 pmol of virus or 30 pmol of the LEGGFYG (SEQ ID NO: 74) epitope, since each virion expresses 60 copies of the epitope.

Example 22

This Example illustrates the protection of relevant animal models from disease caused by *H. influenzae*.

The infant rat model of bacteremia (Loeb et al, 1987) was used to assess the protective abililty of anti-Eagan rTbp1 and anti-Eagan rTbp2 antisera. Anti-Eagan rTbp1 antisera raised in either rabbits or guinea pigs was not protective in this model but anti-Eagan rTbp2 antisera raised in rabbits or guinea pigs was protective (Table 7). These data indicate the use for rTbp2 proteins as protective antigens.

The chinchilla model of otitis media (Barenkamp et al, 1986) was used to assess the protective ability of SB12 rTbp2. Data indicated that compared with the control group, the immunized animals had less severe disease.

The ability of recombinant Tbp2 (rTbp2) to prevent nasopharyngeal colonization was determined in chinchillas. *H. influenzae* strain 12 was grown on supplemented Mueller Hinton agar containing 10-100 $\mu g\ mL^{-1}$ of streptomycin until spontaneous str^R colonies were obtained. Strain 12 resistant to 100 $\mu g\ mL^{-1}$ of streptomycin was used in the colonization model which enabled the specific culture of *H. influenzae* bacteria from nasal ravages.

Chinchillas (3-4 month old or 1-2 year old) were immunized 3 times (i.m.) with either 30 μg of rTbp2 in alum, or $2x10^9$ cfu inactivated strain 12 whole cells in alum, or alum alone on days 0, 14 and 28. On day 44, animals were lightly anesthetized using ketamine HCl. Intranasal inoculations were performed via passive inhalation (50 μL per nare, total 0.1 ml per animal) of freshly cultured streptomycin resistant NTHi strain 12 in BHI medium supplemented with hemin and nicotinamide adenine dinucleotide (NAD). The dose of bacterial challenge was $1x\ 10^8$ cfu per animal.

Nasopharyngeal lavage was performed on days 5 and 9 post inoculation. Secretions were obtained from anesthetized chinchillas by irrigating the nasopharynx with 1 ml of sterile diluent (diluent: 50 mL BHI, 100 mL saline, 200 μg NAD and 25 μL of 1% hemin) and collecting fluid out of the contralateral nares. Normally about 500 μL of fluid was collected from each animal and 25 μL of sample was plated on chocolate agar plate in the presence of 1 mg streptomycin. The results are shown in Table 10. On both 5-day and 9-day post challenge, 25 μL of undiluted nasal lavage fluid were plated in the presence of streptomycin. Although a few animals in the two positive control groups (whole cell immunization and convalescent) had a positive nasopharyngeal colonization on 5 days post challenge, all bacteria were cleared from these animals by day 9, and none of the animals from these two groups developed ear infection. Thus, rTbp2 was shown to partially protect chinchillas against NTHi colonization.

Example 23

This Example describes the generation of truncated analogues of transferrin receptor protein Tbp2.

H. influenzae Tbp2 is produced in low amounts by *E. coli*. The Eagan *tbpB* gene was truncated from its 3'-end using an Erase-a-base kit to produce a number of truncated analogues of Tbp2 as shown in Figure 31 and Table 8. The truncated genes were expressed in *E. coli* BL21(DE3) from the T7 promoter. The percentage of the remaining mature Eagan rTbp2 is indicated and the expression level of the truncated clones is compared with that of the full-length Eagan rTbp2 clone (Table 8). Binding of the truncated clones to human transferrin (Tf) was measured using the assay described by Morton and Williams (1990) (Figure 32). The data indicate that the yield of Eagan rTbp2 can be significantly increased by truncation of the carboxy region of the protein. The data also indicate that the

transferrin binding site may be located between residues 348 and 446 of the mature Eagan Tbp2 protein (Table 3 and Figure 32). However, the transferrin preservation of the Tf binding site in the truncated rTbp2 may not be necessary for its use as a protective immunogen.

The infant rat model of bacteremia (Loeb et al, 1987) was used to assess the protective ability of anti-truncated analogues of transferrin receptor protein Tbp2 and the results are shown in Table 9. Animals were considered to be protected if they had <10cfu in 2 μl of blood.

SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides purified and isolated DNA molecules containing transferrin receptor genes, the sequences of these transferrin receptor genes and the derived amino acid sequences thereof. The invention also provides peptides corresponding to portions of the transferrin receptor. The genes, DNA sequences, recombinant proteins and peptides are useful for diagnosis, immunization and the generation of diagnostic and immunological reagents. Vaccines based upon expressed recombinant Tbp1 and/or Tbp2, portions thereof, or peptides derived from the provided sequences can be prepared for prevention of diseases caused by bacterial pathogens that produce transferrin receptor. Modifications are possible within the scope of this invention.

TABLE 1

leader	1st residue	plasmid	strain
E. coli lpp'	Cys	JB-1360-1R-10	JB-1407-1-1
E. coli lpp	Ser	JB-1366-1R-7	JB-1407-3-1
E. coli pal	Cys	JB-1360-3-10	JB-1407-2-1
E. coli pal	Ser	JB-1366-3R-5	JB-1407-4-4
E. coli rlpB	Cys	JB-1399-1	JB-1437-1-1
E. coli rlpB	Ser	JB-1378-7	JB-1407-5-1

Substitute Sheet (Rule 26)

TABLE 2

PREDICTED ANTIGENIC Tbp1 PEPTIDES

PEPTIDE	RESIDUES[1]	SEQUENCES	SEQ ID NO:
TBP1-N	1-36	AETQSIKDTKEAISSEVDTQSTEDSELETISVTAEK	13
TBP1-2	31-66	SVTAEKVRDRKDNEVTGLGKIIKTSESISREQVLNI	14
TBP1-3	59-94	SREQVLNIRDLTRYDPGISVVEQGRGASSGYSIRGM	15
TBP1-4	88-123	GYSIRGMDRNRVALLVDGLPQTQSYVVQSPLVARSG	16
TBP1-5	117-152	PLVARSGYGTGAINEIEYENVKAVEISKGGSSSEYG	17
TBP1-6	147-182	SSSEYGNGALAGSVTFQSKSAADILEGDKSWGIQTK	18
TBP1-7	179-214	GIQTKNAYSSKNKGFTHSLAVAGKQGGFEGVAIYTH	19
TBP1-8	208-243	GVAIYTHRNSIETQVHKDALKGVQSYDRFIATTEDQ	20
TBP1-9	236-271	IATTEDQSAYFVMQDECLDGYDKCKTSPKRPATLST	21
TBP1-10	266-301	PATLSTQRETVSVSDYTGANRIKPNPMKYESQSWFL	22
TBP1-11	293-328	YESQSWFLRGGYHFSEQHYIGGIFEFTQQKFDIRDM	23
TBP1-12	322-357	KFDIRDMTFPAYLRPTEDKDLQSRPFYPKQDYGAYQ	24
TBP1-13	352-387	DYGAYQHIGDGRGVKYASGLYFDEHHRKQRVGIEYI	25
TBP1-14	383-418	GIEYIYENKNKAGIIDKAVLSANQQNIILDSYMRHT	26
TBP1-15	412-447	DSYMRHTHCSLYPNPSKNCRPTLDKPYSYYHSDRNV	27
TBP1-16	443-478	SDRNVYKEKHNMLQLNLEKKIQQNWLTHQIAFNLGF	28
TBP1-17	469-504	THQIAFNLGFDDFTSALQHKDYLTRRVIATASSISE	29
TBP1-M	498-534	TASSISEKRGEARRNGLQSSPYLYPTPKAELVGGDLC	30
TBP1-19	528-563	LVGGDLCNYQGKSSNYSDCKVRLIKGKNYYFAARNN	31
TBP1-20	558-593	FAARNNMALGKYVDLGLGMRYDVSRTKANESTISVG	32
TBP1-21	588-623	STISVGKFKNFSWNTGIVIKPTEWLDLSYRLSTGFR	33
TBP1-22	618-653	LSTGFRNPSFAEMYGWRYGGKDTDVYIGKFKPETSR	34
TBP1-23	648-683	KPETSRNQEFGLALKGDFGNIEISHFSNAYRNLIAF	35

TABLE 2 (cont)

TBP1-24	677-712	YRNLIAFAEELSKNGTTGKGNYGYHNAQNAKLVGVN	36
TBP1-25	706-741	AKLVGVNITAQLDFNGLWKRIPYGWYATFAYNRVKV	37
TBP1-26	735-770	AYNRVKVKDQKINAGLASVSSYLFDAIQPSRYIIGL	38
TBP1-27	764-799	SRYIIGLDYDHPSNTWGIKTMFTQSKAKSQNELLGK	39
TBP1-28	794-829	NELLGKRALGNNSRNVKSTRKLTRAWHILDVSGYYM	40
TBP1-29	825-854	SGYYMVNRSILFRLGVYNLLNYRYVTWEAV	41
TBP1-30	843-865	LLNYRYVTWEAVRQTAQGAEFDI	42
TBP1-31	42-50	DNEVTGLGK	43
TBP1-32	61-76	EQVLNIRDLTRYDPGI	44
TBP1-33	61-95	EQVLNIRDLTRYDPGISVVEQGRGASSGYSIRGMD	45
TBP1-34	128-146	GAINEIEYENVKAVEISKG	46
TBP1-35	155-161	GALAGSV	47
TBP1-1	1-14	AETQSIKDTKEAISC[2]	48

1. Residue number from the sequence of Tbp1 of *H. influenzae* type b strain Eagan (as shown in Figure 8).

2. Cysteine added to facilitate coupling to a carrier protein, for example KLH.

TABLE 3

PREDICTED CONSERVED ANTIGENIC Tbp2 PEPTIDES

PEPTIDE	RESIDUES[1]	SEQUENCES	SEQ ID NO:
TBP2-1	18-31	CSGGGSFDVDNVSN	49
TBP2-2	231-261	LEGGFYGPKGEELGFRFLAGDKKVFGVFSAK	50
TBP2-3	358-380	TVGKKTYQVEACCSNLSYVKFGM	51
TBP2-4	527-549	ATVKGAFYGPKASELGGYFTYNG	52
TBP2-5	1-36	MKLAALNLFDRNKPSLLNEDSYMIFSSRSTIEEDV	53
TBP2-6	29-64	STIEEDVKNDNQNGEHPIDSIVDPRAPNSNENRHG	54
TBP2-7	57-92	SNENRHGQKYVYSGLYYIQSWSLRDLPNKKFYSGY	55
TBP2-8	85-120	KKFYSGYYGYAYYFGNTTASALPVGGVATYKGTWS	56
TBP2-9	113-148	TYKGTWSFITAAENGKNYELLRNSGGGQAYSRRSA	57
TBP2-10	141-176	AYSRRSATPEDIDLDRKTGLTSEFTVNFGTKKLTG	58
TBP2-11	169-204	GTKKLTGGLYYNLRETDANKSQNRTHKLYDLEADV	59
TBP2-12	197-232	YDLEADVHSNRFRGKVKPTKKESSEEHPFTSEGTL	60
TBP2-13	225-260	FTSEGTLEGGFYGPEGQELGGKFLAHDKKVLGVFS	61
TBP2-14	253-288	KVLGVFSAKEQQETSENKKLPKETLIDGKLTTFKT	62
TBP2-15	281-316	KLTTFKTTNATANATTDATTSTTASTKTDTTTNAT	63
TBP2-16	309-344	DTTTNATANTENFTTKDIPSLGEADYLLIDNYPVP	64
TBP2-17	337-372	IDNYPVPLFPESGDFISSKHHTVGKKTYQVEACCS	65
TBP2-M	360-406	CSNLSYVKFGMYYEAPPKEEEKEKEKDKDKEKEKQ	66
TBP2-19	393-428	KEKDKDKEKEKQATTSIKTYYQFLLGLRTPSSEIP	67
TBP2-20	421-456	TPSSEIPKEGSAKYHGNWFGYISDGETSYSASGDK	68
TBP2-21	449-484	YSASGDKERSKNAVAEFNVNFAEKTLTGELKRHDT	69
TBP2-22	477-512	ELKRHDTQNPVFKINATFQSGKNDFTGTATAKDLA	70
TBP2-23	505-540	ATAKDLAIDGKNTQGTSKVNFTATVNGAFYGPHAT	71

Table 3 (Cont)

TBP2-24	533-559	FYGPHATELGGYFTYNGNNPTDKNSS	72
TBP2-C	553-581	CPTDKNSSSNSEKARAAVVFGAKKQQVETTK	73
TBP2-25	231-238	LEGGFYGP	74
TBP2-26	18-25	CSGGGSFD	75
TBP2-27	130-134	YVYSGL	76
TBP2-28	345-355	CCSNLSYVKFG	77
TBP2-29	401-407	FLLGHRT	78
TBP2-30	450-456	EFNVDF	79
TBP2-31	485-491	NAFTGTA	80
TBP2-32	516-522	VNGAFYG	81
TBP2-33	527-532	ELGGYF	82
TBP2-34	562-566	VVFGAR	83
TBP2-35	562-568	VVFGAK	84
TBP2-36	231-238	LEGGFYG	85

1. Residue number from the sequence of Tbp2 of *H. influenzae* type B Eagan strain (as shown in Figure 9).

TABLE 4

Guinea pig antibody responses to Tbp1 and Tbp2 peptides

PEPTIDE	SEQ ID	SEQUENCES	TITRE
TBP1-N	13	AETQSIKDTKEAISSEVDTQSTEDSELETISVTAEK	500
TBP1-M	30	TASSISEKRGEARRNGLQSSPYLYPTPKAELVGGDLC	1562500
TBP1-1	48	AETQSIKDTKEAISC	<100
TBP2-1	49	CSGGGSFDVDNVSN	2500
TBP2-2	50	LEGGFYGPKGEELGFRFLAGDKKVFGVFSAK	12500
TBP2-3	51	TVGKKTYQVEACCSNLSYVKFGM	62500
TBP2-4	52	ATVKGAFYGPKASELGGYFTYNG	<100
TBP2-M	66	CSNLSYVKFGMYYEAPPKEEEKEKEKDKDKEKEKQA	2500
TBP2-C	73	CPTDKNSSSNSEKARAAVVFGAKKQQVETTK	312500

AMENDED SHEET

TABLE 5

Neutralizing activity of anti-Tbp2 and anti-peptide sera against polio/Tbp2 hybrid viruses

Sera [a]	Neutralizing Titre v. Virus [b]				
	PV1TBP2A	PV1TBP2B	PV1TBP2C	PV1TBP2D	PV1XLD
Rb @PV1	>40,9600	25,844	20,480	16,763	>40,960
Rb 516 D0	<4	<4	<4	<4	<4
Rb 516 D42	40	20	<4	<4	<4
GP561, 562 D0 pool	<4	<4	<4	<4	<4
GP 561 D56	>2048	>2048	>2048	1164	<4
GP 562 D56	>2048	>2048	25	10	<4
GP558, 559, 560 D56 pool	<4	<4	<4	<4	<4

[a] Rb @PV1 is pool of rabbit immune sera raised against PV1XLD. Rabbit 516 was immunised with three successive 3 μg dses of recombinant *H. influenzae* DL63 transferrin binding protein 2 on days 1, 14 and 28. Serum was collected on days 0 (D0) and 42 (D42). Guinea-pigs were immunized with four successive doses of 200μg of peptide on days 1, 14, 28 and 42. Sera were collected on day 0 (D0) and day 56 (D56). Guinea-Pigs 561 and 562 received a peptide containing the sequence LEGGFYGP (SEQ ID NO:74). Guinea-pigs 558, 559 and 556 received a control peptide with an unrelated sequence.

[b] Titre is the inverse dilution of serum giving a 50% endpoint in a virus neutralization assay versus 100 $TCID_{50}$ of virus.

TABLE 6

Peptide-specific IgG titres of rabbits immunised with PV1TBP2A or PV1TBP2B

RABBIT (ANTIGEN)	PEPTIDE-SPECIFIC IgG TITRES [a]	
	PREBLEED	DAY 27
40 (PV1TBP2A)	<20	640
41 (PV1TBP2A)	<20	640
42 (PV1TBP2A)	<20	2560
43 (PV1TBP2B)	<20	160
44 (PV1TBP2B)	<20	1280
45 (PV1TBP2B)	<20	1280
10 (PV1 Mahoney)		<20 [b]
11 (PV1 Mahoney)		<20

[a] Titres are the reciprocal of the greatest dilution of sera giving an A_{450} of at least twice the background value. The background value was the mean A_{450} of wells assayed in the absence of rabbit sera.

[b] Titres for rabbits 10 and 11 refer to sera taken on day 42 after three immunisations with PV1 Mahoney. Rabbits 10 and 11 were immunised as rabbits 40 to 45, except that an additional booster dose was administered on day 28.

TABLE 7 - Infant Rat Protection studies with anti-rTbp1 and anti-rTbp2 antisera

Antisera	# animals protected	# animals infected
rabbit anti-rTbp1	0/10	10/10
pre-bleed	0/10	10/10
rabbit-anti MinnA	10/10	0/10
gp anti-rTbp1	0/10	10/10
pre-bleed	0/10	10/10
gp anti-MinnA	10/10	0/10

Antisera	# animals protected	# animals infected
rabbit anti-rTbp2	8/10	2/10
pre-bleed	0/10	10/10
rabbit anti-MinnA	10/10	0/10
gp anti-rTbp2	10/10	0/10
pre-bleed	0/10	10/10
gp anti-MinnA	10/10	0/10

Antisera were raised in guinea pigs and rabbits to Eagan rTbp1 and Eagan rTbp2 proteins. Infant rats were immunized s.c. with 0.1ml of antisera and 24h later were challenged i.p. with 350cfu of *H. influenzae* type b strain MinnA. Blood was collected 20h post-challenge and plated on chocolate agar.

TABLE 8 - Truncated Eagan rTbp2 clones

Clone	%Tbp2	Expression	Tf binding
	100%	+	+
DS-1461-8-1	98%	ND	ND
DS-1466-1-1	83%	+	+
DS-1466-1-14	80%	+	+
DS-1466-2-6	69%	+	+
DS-1466-3-4	63%	+	+
DS-1466-3-1	62%	+	+
DS-1644-7-9	61%	+	+
DS-1466-1-4	60%	+	+
DS-1457-3-1	54%	++	-
DS-1466-4-1	45%	++	-
DS-1466-5-1	38%	++	-
DS-1466-4-3	16%	ND	ND
DS-1466-1-18	10%	ND	ND

Table 9 - Protection of infant rats by truncated rTbp2

Antiserum	Animals protected[1]	Animals protected[2]
pre-bleed	0/10	0/10
anti-rTbp2 (100%)	7/8	9/10
anti-rTbp2 (83%)	7/8	8/10
anti-rTbp2 (80%)	5/8	10/10
anti-rTbp2 (69%)	3/8	2/10
anti-rTbp2 (60%)	7/8	8/10
anti-rTbp2 (54%)	6/8	6/10
anti-*E. coli*	0/8	0/10
anti-*H. influenzae*	6/6	10/10

1 Rabbit antiserum
2. Guinea pig antiserum

Table 10 - Protective Ability of rTbp2 against NTHi Nasopharyngeal Colonization in Chinchillas

Antigens	# of animals showed positive nasopharyngeal colonization / total # animals	
	5-day post	9-day post
alum	8/10	10/10
rTbp2 + alum	6/10 (ρ=0.628)	6/10 (ρ=0.0433)*
whole cell + alum	3/8 (ρ=0.145)	0/8 (ρ=0.00002)*
convalescent	1/6 (ρ=0.035)	0/6 (ρ=0.0001)*

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